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PATENT COOPERATION TREATY

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To:

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Jerusalem 91342
ISRAEL
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PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	11.07.2000
Applicant's or agent's file reference a645-49-V	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IL99/00184	30/03/1999	07/04/1998

Applicant
STATE OF ISRAEL/MINISTRY OF AGRICULTURE et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	~~Vullo.~~ C **Stefanie Büchler** Tel.+49 89 2399-8061

Form PCT/IPEA/416 (July 1992)

[redacted]ENT COOPERATION TRE[redacted]

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
a645-49-V		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IL99/00184	30/03/1999	07/04/1998
International Patent Classification (IPC) or national classification and IPC		
C12N15/40		
Applicant		
STATE OF ISRAEL/MINISTRY OF AGRICULTURE et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
28/10/1999	11.07.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Petri. B Telephone No. +49 89 2399 7356

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL99/00184

I. Basis of th report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-16 as originally filed

Claims, No.:

1-20 as received on 01/06/2000 with letter of 01/06/2000

Drawings, sheets:

1/1 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

see separate sheet

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventiv step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	7-11, 15
	No:	Claims	1-6, 12-14, 16-20
Inventive step (IS)	Yes:	Claims	7
	No:	Claims	8-11, 15
Industrial applicability (IA)	Yes:	Claims	1-20
	No:	Claims	none

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: Huet et al. 1994 J. Gen. Virol. 75:1407-1414; XP-002 118 196;
D2: Embl. Accession X77756; XP-002 118 197;
D3: Lecoq et al. 1991 Plant Disease 75:208-211; XP-002 118 201;
D4: Gal On et al. 1992 J. Gen. Virol. 73:2183-2187; XP-002 118 198;
D5: Granier et al. 1993 J. Gen. Virol. 74:2737-2742; XP-002 118 202;
D6: WO 95/12669;

Novelty; Art 33(2), PCT

1) The subject-matter of claims 1-6, 12-14, 16-20 is not novel (Article 33(2) PCT).

1.1) D1 discloses recombinant potyvirus infectious nucleic acid constructs, comprising a full length clone (pZYMK-HC(GI-T)) characterized in that its HC-Pro gene conserved FRNK box sequence contains a substitution (pZYMK-HC(GI-T) (Fig. 1b, last bar).

Irrespective of the question whether pZYMK-HC(GI-T) were known to be useful in plant cross protection, said feature is inherent to above hybrid strain pZYMK-HC(GI-T) as its HC-Pro gene comprises a substitution in the FRNK box, i.e. Arg^{180} to Ile^{180}, which, as has been shown by the applicant, confers usefulness in plant cross protection.

In addition, the wording *"characterized only"* does not exclude that the full-length clone contains in addition substitutions at any other position of the virus.

Consequently subject-matter of claim 1 lacks novelty in view of the full length clone pZYMK-HC(GI-T) of D1.

1.2) As the full length clone pZYMK-HC(GI-T) of D1 comprises inserted sequences of DNA or RNA (e.g. the T7 promotor) the constructs of claim 12 also lack novelty.

1.3) As the full length clone pZYMK-HC(GI-T) of D1 is used for inoculating plants and to obtain progeny viruses, the methods of claim 13-14 and 16, the virus of claim 17 the produce of claim 18-19 and the composition of claim 20 also is considered to lack novelty in view of D1.

2.) Subject-matter of claim 7-11, 15 is considered novel.

2.1) As the HC-Pro sequence of full length clone of D1 differs from the sequence of ZYMV-AG1 of present claim 1 at position 148 (pZYMK-HC(GI-T):Gly148 vs. ZYMV-AG1:Asp148) subject-matter of claim 7 is considered to be novel.

2.2) Although strain ZYMK-HC(-) comprises a further mutation which effectively abolishes aphid transmissibility (i.e. the mutation at pos 308, see D1 Table 2) subject-matter of claim 8 is considered novel, as apparently isolate of strain ZYMK-HC(-) was not available as infectious nucleic acid full length clone.

2.3) Although the mutation at position 10 of the DAG triplet of the CP locus and its effect on aphid transmissibility is known from D4, subject-matter of claim 9 is considered novel as neither D1 nor D4 show infectious full length clones of potyvirus which comprise both mutations.

2.4) As subject-matter of claim 7 is novel, subject-matter of dependent claim 10 is also considered novel.

2.5) As D1 comprises only isolates and full length clones of ZYMV, subject-matter of claims 11 and 15, which is restricted to different potyviruses, is not disclosed by D1 and thus considered novel.

Inventive Step; Art. 33(3), PCT

3.) The subject-matter proposed in claims 8-11, 15 of the present application cannot be considered as involving an inventive step (Article 33(3) PCT) for the following reasons:

3.1) Inclusion of the substitution of the Ala^{10} in the conserved DAG triplet in the N terminal

region of CP into the context of the present application lacks an inventive step, since its effect, namely the cause of loss of aphid transmissibility is known from D4. As the technical effect of said additional substitution is restricted to the known phenotype caused by that substitution, i.e. said loss of aphid transmissibility, it would be obvious to the person skilled in the art to include this feature into any strain ZYMK, in order to obtain aphid intransmissibility (see also PCT Gazette IV-8.8 A1 (iii)).
Consequently subject-matter of claims 8-10 is considered not to be based on an inventive step

3.2) Given the high degree of similarity among members of the potyvirus group, the extrapolation of the known subject-matter of claim 1-6 (see item 1) to other such closely related viruses is not considered to involve an inventive step. As such claim 11, 15 are considered not to comprise an inventive contribution to the art.

4) Subject-matter of claim 7 and any subject-matter dependent thereof is considered to involve an inventive step.

D1 is considered to represent the closest prior art, and discloses an infectious nucleic acid full length clone of a potyvirus i.e. pZYMV-HC(GI-T), characterized in that the amino acid residue of the HC-Pro gene at position 148 is Gly and at position 180 is Ile. The subject-matter of present claim 7 differs from said closest prior art in that a different full length clone of potyvirus is provided i.e. ZYMV-AG1 with Asp at position 148 and Ile at position 180 of said gene.

The technical effect of said difference is that a strain is provided which is *"useful in cross protection"*.

As such a technical effect is already known from D3 and is inherent to pZYMV-HC(GI-T) of D1, the technical problem to be solved may be considered as to provide alternative strains which are *"useful in plant cross protection"*.

As none of the available prior art disclosures indicated a link between the *"usefulness in plant cross protection"* and mutations in the FNRK box of the HC-Pro locus, the specific construct as depicted in Fig. 1 d) is considered inventive.

Re Item VIII
Certain observations on the international application

5. Claim 7 does not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. Construct/strain denomination ZYMV-AG1 is arbitrary and thus meaningless to the skilled person. The technical features characterising said construct/strain need to be included into the claim (see however below).

In said context it is to be noted that although said strain may be characterized in the description, in order to fulfill the requirements of Art. 6, the claim itself has to contain all the necessary technical features characterizing the subject-matter for which protection is thought.

6. The above lack of novelty (item 1), seems mainly to be due to the wording of claim 1:

The Examining Division recognizes the applicants finding that out of the three known mutations in the HC-Pro gene of the mild and poorly aphid transmissible strain ZYMK-WK as disclosed in D1 (i.e. Asp148 to Gly148, Arg180 to Ile180, Thr308 to Ala308) a single substitution, i.e. Ile180, is sufficient to cause the "mild" phenotype and to render strains of potyvirus "usefull in plant cross protection".

However, the scope of present claim 1 comprises any potivirus full length clone that shows a substitution in the FRNK motiv of the HC-Pro locus (as neither the wording *characterized in*, nor *characterized **only** in*, excludes additional features such as amino acid variation at other positions). In view pZYMV-HC(GI-T) of D1 any claim to a full length clone not restricted to such clones which do not exhibit the other mutations comprised in HC-Pro of pZYMV-HC(GI-T) of D1 lacks novelty.

In any case, the general term *"substitution"* without a reference sequence does not pose any limitation to the scope. This seems particularly true for viral genes where several isolates with varying sequences are known (see D1 Fig. 2) with no apparent wild type sequence.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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C12N 15/40, 15/57, 15/82, 15/83, 7/04, 7/00, A01N 63/02

A3

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(30) Priority Data:
123994 7 April 1998 (07.04.98) IL

(71) Applicant (*for all designated States except US*): STATE OF ISRAEL/MINISTRY OF AGRICULTURE [IL/IL]; Agricultural Research Organization, The Volkani Center, 50250 Bet Dagan (IL).

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(74) Agent: NOAM, Meir; P.O. Box 34335, 91342 Jerusalem (IL).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
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Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
9 December 1999 (09.12.99)

(54) Title: RECOMBINANT POTYVIRUS CONSTRUCT AND USE THEREOF

(57) Abstract

The present invention relates to a recombinant potyvirus infectious nucleic acid, preferably cDNA or RNA, construct useful for plant cross protection, comprising a full length clone characterized in that its HC-Pro gene conserved FRNK box sequence contains a substitution, preferably a substitution of Arg. Preferably, the construct further contains a substitution which effectively abolishes aphid transmissibility. The present invention further relates to a method for the production of a mild strain of potyvirus utilizing the above mentioned construct and to a method for protecting plants from viral infection and for transient expression of foreign nucleic acid (genes) in plants, using said construct. Preferably, the present invention relates to a method for cross protection of curcurbits against ZYMV infection. The present invention also relates to a method for providing protection against viral infection in plants comprising inoculating plants, by mechanical inoculation or by bombardment, with the recombinant potyvirus infectious construct.

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INTERNATIONAL SEARCH REPORT

Application No
PCT/IL 99/00184

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/40 C12N15/57 C12N15/82 C12N15/83 C12N7/04
C12N7/00 A01N63/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUET, H., ET AL: "Mutations in the helper component protease gene of zucchini yellow mosaic virus affect its ability to mediate aphid transmissibility" JOURNAL OF GENERAL VIROLOGY, vol. 75, 1994, pages 1407-1414, XP002118196 cited in the application the whole document	1-11, 17-20
Y	-& HUET, H., ET AL.: "Zucchini yellow mosaic virus (ZYMV) HC-PRO gene" EMBL ACCESSION NO: X77756, 25 April 1994 (1994-04-25), XP002118197 the whole document	8,12-16

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
13 October 1999	27/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	GAL-ON, A., ET AL.: "A zucchini yellow mosaic virus coat protein gene mutation restores aphid transmissibility but has no effect on amplification" JOURNAL OF GENERAL VIROLOGY, vol. 73, 1992, pages 2183-2187, XP002118198 cited in the application the whole document	8
Y	MAIA, I.G., ET AL.: "Potyviral HC-PRO: a multifunctional protein" JOURNAL OF GENERAL VIROLOGY, vol. 77, 1996, pages 1335-1341, XP002118199	12
A	the whole document	1-11, 13-20
Y	FUCHS, M., ET AL.: "Management of virus diseases by classical and engineered protection" MOLECULAR PLANT PATHOLOGY ON-LINE 'HTTP://WWW.BSPP.ORG.UK/MPPOL/! 1997/0116FUCHS, 'Online! 1997, pages 1-19, XP002118200 Retrieved from the Internet: <URL:http://www.bspp.org.uk./mppol/1997/0116fuchs/> 'retrieved on 1999-10-08! see section 2.3 pages 5-6	13,14
Y	WO 95 12669 A (TEXAS A & M UNIVERSITY SYST) 11 May 1995 (1995-05-11) the whole document	15
Y	LECOQ, H., ET AL.: "Control of zucchini yellow mosaic virus in squash by cross protection" PLANT DISEASE, vol. 75, 1991, pages 208-211, XP002118201 the whole document	16

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	GRANIER, F., ET AL.: "Mutations in zucchini yellow mosaic virus helper component protein associated with loss of aphid transmissibility" JOURNAL OF GENERAL VIROLOGY, vol. 74, 1993, pages 2737-2742, XP002118202 the whole document -& GRANIER, F., ET AL.: "Helper component - zucchini yellow mosaic virus (strain E15-PAT)" PIR ACCESSION NO:JQ2396, 30 September 1993 (1993-09-30), XP002118203 the whole document	1-20
A	LECOQ, H., ET AL.: "Characterization of a zucchini yellow mosaic virus isolate with a deficient helper component" PHYTOPATHOLOGY, vol. 81, 1991, pages 1087-1091, XP002118204 the whole document	1-20
A	PENG, Y.-H., ET AL.: "Mutations in the HC-Pro gene of zucchini yellow mosaic potyvirus: effects on aphid transmission and binding to purified virions" JOURNAL OF GENERAL VIROLOGY, vol. 79, April 1998 (1998-04), pages 897-904, XP002118215 the whole document	1-20
A	BLANC, S., ET AL.: "A specific interaction between coat protein and helper component correlates with aphid transmission of a potyvirus" VIROLOGY, vol. 231, 1997, pages 141-147, XP002118205 the whole document	8
A	LIVNEH, ORNA., ET AL.: "Plants transformed with the first (nonstructural) three cistrons of potato virus Y are resistant to potato virus infection" TRANSGENICS (1995), 1(5), 565-71, XP002118206 the whole document	13

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropnate, of the relevant passages	Relevant to claim No.
A	GAL-ON, A., ET AL.: "Particle bombardment drastically increases the infectivity of cloned DNA of zucchini yellow mosaic potyvirus" JOURNAL OF GENERAL VIROLOGY, vol. 76, 1995, pages 3223-3227, XP002118207 the whole document	14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IL 99/00184

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9512669 A	11-05-1995	US 5491076 A	13-02-1996
		AU 1408095 A	23-05-1995
		US 5766885 A	16-06-1998
		ZA 9408561 A	30-06-1995

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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A2

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(30) Priority Data:
123994 7 April 1998 (07.04.98) IL

(71) Applicant *(for all designated States except US)*: STATE OF ISRAEL/MINISTRY OF AGRICULTURE [IL/IL]; Agricultural Research Organization, The Volkani Center, 50250 Bet Dagan (IL).

(72) Inventor; and
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(74) Agent: NOAM, Meir; P.O. Box 34335, 91342 Jerusalem (IL).

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Published
Without international search report and to be republished upon receipt of that report.

(54) Title: RECOMBINANT POTYVIRUS CONSTRUCT AND USE THEREOF

(57) Abstract

The present invention relates to a recombinant potyvirus infectious nucleic acid, preferably cDNA or RNA, construct useful for plant cross protection, comprising a full length clone characterized in that its HC-Pro gene conserved FRNK box sequence contains a substitution, preferably a substitution of Arg. Preferably, the construct further contains a substitution which effectively abolishes aphid transmissibility. The present invention further relates to a method for the production of a mild strain of potyvirus utilizing the above mentioned construct and to a method for protecting plants from viral infection and for transient expression of foreign nucleic acid (genes) in plants, using said construct. Preferably, the present invention relates to a method for cross protection of curcurbits against ZYMV infection. The present invention also relates to a method for providing protection against viral infection in plants comprising inoculating plants, by mechanical inoculation or by bombardment, with the recombinant potyvirus infectious construct.

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AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

RECOMBINANT POTYVIRUS CONSTRUCT AND USE THEREOF

Field of the Invention

The present invention generally relates to a recombinant potyvirus infectious nucleic acid construct useful for providing protection against viral infection in plants and to a recombinant virus harboring said construct. More specifically, the present invention relates to a recombinant potyvirus infectious construct containing an HC - Pro gene whose sequence coding for the conserved FRNK box contains a substitution. Preferably, the Arginin (Arg) is substituted with Isoleucine (Ile).

The present invention further relates to a method for the production of a mild strain of potyvirus utilizing the above mentioned construct and to a method for protecting plants from viral infection and for transient expression of foreign nucleic acid (genes) in plants, using said construct. Preferably, the present invention relates to a method for cross protection of curcurbits against ZYMV infection.

Background of the Invention

The Curcurbitaceae is a broad botanical family comprising several economically important species cultivated worldwide, such as cucumber, squash, cantaloupe, zucchini pumpkin, melon and watermelon. Curcurbit production throughout the world is impaired by several aphid transmitted viruses, the most prevalent being the two potyviruses ZYMV (Zucchini Yellow Mosaic Virus) and WMV-2 (Watermelon Mosaic Virus 2) and CMV (cucumber Mosaic Virus). ZYMV infected plants show symptoms such as vein clearing followed by a yellow mosaic on the infected systemic leaf and may show stunting and distortion. In mild cases of infection the quantity and quality of the yield are damaged and in severe infections there might be a total loss of the yield, causing significant economical losses.

Control measures include phytosanitation, the use of colored plastic mulches for attracting virus bearing aphids and creating a hydrophobic barrier around the plant such as oil sprays. These provide temporary protection and are a limited protection during a massive infection.

Development of virus resistant cultivars either by classical breeding or by introducing viral derived nucleic acid sequences into the plant genome through genetic engineering of plants, is also employed for the protection of plants against virus infection. Squash hybrid transgenic inbred lines exhibiting resistance to ZYMV were produced (Tricoli D.M., Carney K.J., Russell McMaster P.F., Groff D.W., Hadden K.C., Himmel P.T., Hubbard J. P., Boeshore M.L. and Quemada H.D. (1995) *Biotechnology* vol. 13;1458) but these are limited to one cultivar only.

The phenomenon of cross protection, which is the use of a mild strain of a virus to protect against the damage by infection with severe strains of the same virus, provides a good method for controlling virus diseases.

In curcurbits, cross protection, specifically against ZYMV, is an attractive control option. Cross protection is highly effective under severe disease pressure. The severity of the disease conferred by the ZYMV on curcurbits and the latter's relatively short crop cycle (8 - 16 weeks) make cross protection a preferred control option for curcurbits.

The currently used mild strain of ZYMV for cross protection of curcurbits, was obtained by Lecoq (Lecoq H., Lemaire JM., Wipf-Scheible C., (1991) *Plant Dis.* 75:208-211). This strain is designated ZYMV-WK and is poorly transmitted by aphids, causes only mild leaf mottling and does not induce fruit malformation in curcurbits. Plants are inoculated at an early stage with the mild strain (ZYMV-WK), usually by mechanical inoculation.

No full length infectious clone of this mild virus exists.

Potyviruses have a genome consisting of a positive - sense single stranded RNA possessing a covalently linked 5' - terminal viral protein (Vpg) and a 3' terminal poly (A) tail. The viral RNA is expressed as a single polyprotein,

which is subsequently processed by three virus encoded proteases, producing eight to ten genes, which are a conserved region throughout the potyvirus genome. The potyviruses are transmitted from plant to plant by aphids in a non persistent manner, and this process is dependent on the presence of two virus encoded proteins, the coat protein (CP) and the helper component proteinase HC-Pro. The HC-Pro is a multifunctional protein involved in aphid transmission, polyprotein processing, virus replication, symptom expression and in virus movement in the plant (Maia I. G., Haenni A., and Bernardi F., (1996) *Journal of General Virology* 77:1335-1341).

Zucchini yellow mosaic virus (ZYMV) is a member of the potyvirus group which causes devastating epidemics in commercial curcurbits world wide. A full length clone of ZYMV, from which infectious transcripts were produced, was constructed (Gal On A., Antignus Y., Rosner A., and Raccah B. (1991) *Journal of General Virology* 72:2639-2643).

It was found that a substitution of the Alanin (Ala) residue to Threonin (Thr) at position 10 in the conserved DAG (Aspartate - Alanin - Glycine; Asp-Ala-Gly) triplet in the N terminal region of the CP effectively abolished aphid transmissibility of ZYMV (Gal On A., Antignus Y., Rosner A., and Raccah B. (1992) *Journal of General Virology* 73:2183-2187). Also substitution of Thr by Ala at position 309 in the HC-Pro gene of the infectious clone of ZYMV effected aphid transmissibility without changing virus accumulation and symptom development (Huet H., Gal On A., Meiri E., Lecoq H. and Raccah B.(1994) *Journal of General Virology* 75:1407-1414), though less effectively than the substitution in the DAG triplet in the CP of the ZYMV.

It has surprisingly been found that an amino acid substitution in the conserved FRNK box of the potyvirus HC-pro gene allows for the construction of an infectious potyvirus construct, which, when introduced to plants, induces little or no symptom development, and which does not effect the accumulation of the virus in the plant. This infectious construct is therefore a unique potyvirus construct which is highly superior for plant cross protection and for transient

expression of foreign nucleic acid in plants. It has an improved ability of protection against infection by the severe strain of ZYMV, over any of the existing protection methods, is significantly safer and more environment friendly than the naturally occurring viruses, does not cause the development of symptoms in a variety of curcurbits, and is stable (no revertant virus has been found after several passages through plants).

Summary of the Invention

The present invention relates to a recombinant potyvirus infectious nucleic acid, preferably cDNA or RNA, construct useful for plant cross protection, comprising a full length clone characterized in that its HC- Pro gene conserved FRNK box sequence contains a substitution, preferably a substitution of Arg.

Preferably, the construct further contains a substitution which effectively abolishes aphid transmissibility, such as a substitution of the Ala residue at position 10 in the conserved DAG triplet in the N terminal region of the CP or substitution of Thr at position 309 in the HC - pro in ZYMV.

The recombinant construct of the present invention may be useful for plant cross protection (especially against severe strains of ZYMV) and for the transient expression of foreign nucleic acid in plants wherein the full length clone has, in any position, a sequence of DNA or RNA inserted into it or wherein any viral genes are removed from the full length clone (defective RNA). The full length clone may be of any potyvirus, preferably of ZYMV.

The present invention also relates to a method for providing protection against viral infection in plants comprising inoculating plants, by mechanical inoculation or by bombardment, with the recombinant potyvirus infectious construct.

The present invention also relates to a method for introducing foreign nucleic acid into plants according comprising infecting a plant with a full length clone or co-infecting a plant with a full length clone, from which any viral genes are

removed, together with a full length clone or virus harboring a full length clone.

The present invention also relates to a method for the production of a mild strain of potyvirus comprising inoculating plants with the recombinant potyvirus infectious construct as defined in any of the preceding claims and collecting the resulting progeny, and to a virus produced in this method.

The present invention further relates to compositions for plant inoculation or for transient expression of foriegn nucleic acid in plants containing, as an active ingredient, the recombinant construct or a virus containing the recombinant construct.

Detailed Description of the Invention

The present invention relates to a recombinant potyvirus infectious nucleic acid construct useful for plant cross protection and for the transient expression of foreign nucleic acid in plants. The present invention further relates to compositions for plant inoculation or for transient expression of foriegn nucleic acid in plants containing, as an active ingredient, the recombinant construct or a virus containing the recombinant construct.

The construct of the present invention comprises a full length potyvirus clone containing a substitution in the conserved FRNK box sequence in the HC - pro gene, preferably, Arg (in the FRNK box) is substituted with an amino acid having a bulky side chain or an amino acid from the hydrophobic group such as Ile. This substitution in the FRNK box dramatically effects the severity of symptom development without effecting the accumulation of the virus in the plant. Preferably, the construct of the present invention also contains a substitution which effectively abolishes aphid transmissibility , such as the substitution of the Ala residue to Thr at position 10 in the conserved DAG (Asp-Ala-Gly) triplet in the N terminal region of the CP or substitution of Thr by Ala at position 309 in the HC - pro of ZYMV.

Full length infectious clones of a severe strain of ZYMV were constructed and put under the control of a phage promoter, such as the T7 RNA polymerase promoter (Gal On A., Antignus Y., Rosner A., and Raccah B. (1991) *Journal of General Virology* 72:2639-2643), bacterial promoters or a promoter effective *in planta,* such as the cauliflower mosaic virus (CaMV) 35S promoter (Gal On A., Meiri E., Huet H., Hua W.J., Raccah B. and Gaba V. (1995) *Journal of General Virology* 76:3223-3227).

In the work presented here, the FRNK box is implicated, for the first time, as being of importance in symptom development, surprisingly without effecting the accumulation of the virus in the plant. Due to the highly conserved sequence of the FRNK box within the HC -Pro gene of the potyviruses, any substitution in the FRNK box of a potyvirus would have an effect on symptom development, not only the substitution of Arg in position 180 with Ile, in ZYMV, demonstrated in the work described here.

Based on the highly conserved genome, organization and gene function of the potyviruses, it may be concluded that the conserved FRNK box in the HC - pro gene has the same function in all potyviruses (perhaps as a receptor). Therefore, the substitution in the FRNK box in any of the potyviruses would have a similar effect on symptom development. Members of the potyviruses that are economically important are, for example, BCMV (Bean Common Mosaic Virus), BYMV (Bean Yellow Mosaic Virus), BtMV (Beet mosaic), MWMV (Moroccan watermelon mosaic), OYDV (Onion yellow dwarf), PRSV (Papaya ringspot), PStV (Peanut stripe), PepMoV (Pepper mottle), PVMV (pepper veinal mottle), CGVBV (Cowpea green vein banding), GEV (ground eyespot), ISMV (Iris severe mosaic), JGMV (Johnsongrass mosaic), LYSV (Leek yellow stripe), LMV (Lettuce mosaic), MDMV (Maize dwarf mosaic), PPV (Plum box), PVA (Potato A), PVV (Potato V), PVY (Potato Y), SbMV (Soybean mosaic), SCMV (Sugarcane mosaic), SPFMV (Sweet potato feathery mottle), TEV (Tobacco etch), TVMV (Tobacco vein mottling), TBV (Tulip

breaking), TuMV (Turnip mosaic), WMV-2 (Watermelon Mosaic Virus 2) , YMV (Yam mosaic), ZYFV (Zucchini yellow fleck).

The infectious clone may be an RNA transcript or a cDNA construct, though the use of infectious transcripts is the less efficient process *in vitro*.

A method for providing protection against viral infection in plants, according to the present invention comprises inoculating plants with the recombinant potyvirus infectious construct, for example, by mechanical inoculation or by bombardment.

Compositions containing, as an active ingredient, the construct of the present invention may be used for superior plant cross protection, especially against infection by the severe strain of ZYMV and for transient expression of foreign nucleic acid in plants. The composition used for the introduction of the construct into plants, for infecting them by bombardment is an aqueous composition comprising, in aproximately equal volumes, the construct, a salt, such as calcium nitrate and particles such as tungsten, gold. The composition used for the introduction of the construct into plants by mechanical inoculation comprises infected plant tissue.

The construct of the present invention may be further used as a vehicle for the transient expression of foreign nucleic acid, namely genes, in a plant. The construct according to the present invention is highly infective, does not induce symptoms in the infected plants and is not transmitted by aphids.

Use of compositions, containing as an active ingredient, this clone provides an efficient, safe and environment friendly method for transient expression of foreign nucleic acid into the infected plants. Further applications of this construct may, therefore, be the expression of foreign sequences or genes within a defective RNA molecule of potyviruses. Defective RNAs are viral RNA genomes which are missing some of the viral genes but which, together with a complete helper virus (the full length parental virus), can facilitate the expression of the sequences they contain. Defective RNAs are derived from the helper virus genome, but still require the presence of a complete helper

virus for replication in the plant cell. The construct of the present invention may have viral genes removed from the full length clone and may then serve to support the expression of foreign genes via potyviruses defective RNA by co-infection of a plant with a full length clone, from which any viral genes are removed, together with a full length clone or virus harboring a full length clone.

A method for introducing foreign nucleic acid into plants according to the present invention comprises infecting a plant with a full length clone into which any sequence of DNA or RNA is inserted or co-infecting a plant with a full length clone, from which any viral genes are removed, together with a full length clone or virus harboring a full length clone.

A method for the production of a mild strain of potyvirus, according to the present invention comprises inoculating plants with the recombinant potyvirus infectious construct and collecting the resulting progeny.

The said invention will be further described and illustrated by the following experiments and figure. These experiments and figure do not intend to limit the scope of the invention but to demonstrate and clarify it only.

Brief Description of the Figure

Figure 1 is a schematic drawing of four constructs of ZYMV infectious cDNA (a-d).

Detailed Description of the Figure

Figure 1 is a schematic drawing of four constructs of ZYMV infectious cDNA (a-d). The open and striped bars indicate the ZYMV-NAT and ZYMV-WK sequences within the FLC respectively. The relevant restriction enzymes and the amino acid changes are present. On the right side the severity of the symptoms in squash is indicated, from very severe (+++++) to mild (+). The sequence of the primer used for the mutagenesis is indicated.

Example 1 - full length clone (FLC) of ZYMV

Construction of the mutants in the full length clone (FLC) of ZYMV

The constructs which represent the HC - Pro sequences (Huet H., Gal On A., Meiri E., Lecoq H. and Raccah B.(1994) *Journal of General Virology* 75:1407-1414) of the ZYMV - WK strain were placed under the T7 RNA promoter in the infectious FLC. In order to get higher rate of infection with those constructs the fragment BstXI/AgeI from the FLC of 35SZYMVNOS cDNA (Gal On A., Antignus Y., Rosner A., and Raccah B. (1991) *Journal of General Virology* 72:2639-2643 and Gal On A., Meiri E., Huet H., Hua W.J., Raccah B. and Gaba V. (1995) *Journal of General Virology* 76:3223-3227), was replaced by the appropriate fragment from pZYHC (-) clone (Huet H., Gal On A., Meiri E., Lecoq H. and Raccah B.(1994) *Journal of General Virology* 75:1407-1414). Site directed mutagenesis was introduced on ssDNA template of the subclone pksM16B (Gal On A., Antignus Y., Rosner A., and Raccah B. (1991) *Journal of General Virology* 72:2639-2643), using the primer 5' ATGTTCATAAATAAGCGCTCTAG3' (amino acid Ile is underlined and the unique restriction site of Eco47III is in bold). The clone pksM16B carrying the mutations was double digested by BamHI/BstEII and the obtained fragment (1.4kb) was introduced to the same sites in the 35SZYMVNOS cDNA (Gal On A., Meiri E., Huet H., Hua W.J., Raccah B. and Gaba V. (1995) *Journal of General Virology* 76:3223-3227).

Plants, mechanical or bombardment inoculation and symptom appearance of the ZYMV AG1

Greenhouse - grown zucchini squash (*Curcurbita pepo.* L. cv Ma'ayan), cucumber (*Cucumis sativus* L. cv. Bet Alpha; Shimshon; Delila), melon (*Cucumis melo* L. cv. Arava) and watermelon (*Citrullus lanatus* Schad cv. Malali) plants were used at the cotyledon stage. The inoculated plants were maintained in a growth chamber under continuous light at about 25^0C. The plants were examined daily for visual symtom development.

Bombardment inoculation were as previously described by Gal - On et al. (1995). Mechanical inoculation of plants infected by the recombinant virus were performed by sap inoculation (100mg/ml), applied to a cotyledon previously dusted with carborundum.

Cross protection experiments

Cross protection by the ZYMV-AG1 strain was tested as described by Lecoq et al. (1991) Squash seedlings at the fully expanded cotyledon stage were bombarded with the 35S-AG1 at 0.1 μg/μl. A week later they were infected in the greenhouse by 5 - 7 aphids (*Myzus persicae*) per plant according to Antignus Y., Raccah B., Gal - On A. and Cohen S. (1989) *Phytoparasitica* 17:287-289).

Determination of the mutation in the progeny virions

To ascertain the presence of the mutations in the viral RNA total mRNA from infected leaf tissue was extracted. The synthesis of the RT-PCR was performed as described by Huet et al. (1994).

ELISA assay for evaluation of ZYMV titer

Leaf discs of squash and cucumber ZYMV-infected plants were taken 7 - 10 d.p.i. and the homogenized tissue were subjected to ELISA as described by Antignus et al (1989).

Previously, sequence comparison has shown four amino acid changes in the 455 amino acid sequence of the HC - pro gene between the severe field strain (ZYMV - JV) and the mild field strain ZYMK - WK. The replacement of a fragment of the HC - Pro of ZYMV - WK containing two substitutions Aspartate (Asp) 148 and Arg 180 (BstXI/BstEII fragment), reduced symptom expression of the virus in squash plants without effecting virus accumulation. To distinguish which of the two substitutions, Asp 148 or Arg 180, effect

symptom development, Arg 180 was replaced by Ile within the FRNK box (figure 1, clone d) by site directed mutagensis.

The engineered virus containing the Arg 180 replacement by Ile, was designated ZYMV-AG1. This new strain did not cause the development of symptoms in cucumber (three different varieties), melon and watermelon. The virus did accumulate to levels as high as that of the wild type ZYMV-JV. It was assumed, therefore, that the second amino acid difference (Asp at position 148) is dispensable for altering the symptoms from mild to severe.

In order to verify the presence of the amino acid changes within the mild virus ZYMV - AG1, and to prevent aphid transmission, a new restriction site of Eco47III was introduced at position 550 nt (from the 5' of the HC- Pro gene) and the DAG motif in the CP was replaced by DTG respectively (figure 1).

The new engineered virus (AG1) and a wild type severe strain (JV) accumulated to a similar level in systemically infected leaves of different curcurbit species (Table 1). Therefore, it may be concluded, that a point mutation changing amino acid Arg 180 to Ile, dramatically effects the severity of symtom development without effecting the movement and the replication of the ZYMV virus in the plant. The dramatic results confered by a point mutation in the potyvirus FRNK box, demonstrated in this work for the first time, could not have been inferred from the mere known sequence comparison which showed amino acid changes between the severe field strain and the mild field strain.

The stability of the amino acid substitution Arg 180 to Ile within ZYMV-AG1 was tested by infecting hundreds of squash plants and dozens of cucumber plants (Table 2). The presence of the Ile 180 mutation in the HC - Pro was confirmed by sequencing (data not shown). Curcurbit plants inoculated with ZYMV-AG1 mechanically or by particle bombardment with the ZYMV-AG1 strain did show the mild symptom appearance even throughout the growing period of the plant (Table 2). The presence of the Ile 180 mutation within the

virion genome was confirmed by sequencing or indirectly by digestion of the RT-PCR amplified fragment with the restriction enzyme Eco47III (figure 1). Replication and movement of the engineered ZYMV-AG1 strain remained high (as the wild type ZYMV), as seen from the accumulated level of the virus. These results suggest that no selective pressure is exerted to cause a reversion in the virus mutated genome.

The ability of the newly produced mild strain (ZYMV-AG1) to protect against a challenge inoculation of the severe strain of ZYMV (JV), was studied in cross protection experiments. Most of the protected plants did show mild symptoms after a challenge with the severe strain (96% protection). Two plants out of 47 that were infected with the ZYMV-AG1 strain and challenged a week later with the JV strain exhibited severe symptoms about one month post inoculation (Table 3).

The protection was studied in a small field experiment in which protected plants were exposed to field inoculation. Approximately 40% of the control non-protected plants became infected, while none of the protected plants showed severe symptoms. Therefore, no fruit damage was observed in the protected plants (Table 3). Previous studies showed that in a typical cross protection phenomenon, both the protective and the challenge virus strains are very closely related (Perring T.M., Farrar C. A., Blua M. J., Wang H.L. and Gonsalves D. (1995) *Crop Protection* 14 no. 7, 601 - 606). This is the first report where cross protection takes place between strains that have an identical sequence, including the coat protein sequence, that differ only in a single amino acid in a non structural protein (the HC - Pro).

2) Cross protection in melons

Melon (*Cucumis melo* L. cv. Ofir) seedlings were planted and were infected with ZYMV-WK and the recombinant virus ZYMV-AG1. The viruses were sprayed onto the melon seedlings prior to planting. The seedlings were then planted together with untreated (control) seedlings.

Half of the plants at three weeks were challenged mechanically with the wild type virus (ZYMV-JV) and half were unchallenged for testing natural infection.

30 days after the begining of the experiment parameters such as the plant size and the extent of infection with the wild type virus, were studied. Plants infected with ZYMV-JV that were not treated by the weakened virus (WK) were small and showed clear infection symptoms. Plants treated with the recombinant virus (ZYMV - AG1) showed no symptoms of infection.

3) Expression of foreign genes through the ZYMV-AG1 clone in plants

For the expression of a foriegn gene in an infected plant, a Pst I site was inserted into the ZYMV-AG1 between the NIb and CP genes. The GFP (green flurocent protein) reporter gene and the Bar gene, which confers resistance to the non selective herbicide bialaphos (commercially named BASTA), were amplified by PCR, using primers containing the Pst I restriction site, and were inserted in the PstI site.

Plants were inoculated by bombardment with the ZYMV - AG1 containing the GFP reporter gene or Bar gene.

Biochemical analysis showed the GFP and Bar gene to be highly and stably expressed. Even after several passages, no revertants of the recombinant mild virus were found and the reporter gene and Bar expression remained high and stable. Plants expressing the GFP were luminecent and plants expressing the Bar gene were found resistant to the herbicide bialaphos.

Table 1. Comparison of virus accumulation between ZYMV-JV and ZYMV-AG1 strains in cucurbits.

experiment no.	no of tested plants: JV, AGI, WK	ZYMV-JV# severe ELISA OD(405)	ZYMV-AG1^ mild	ZYMV-WK~ mild
1s+	11, 6, 6	0.9* (0.41)**	0.5 (0.19)	0.7 (0.18)
2s	2, 9, 8	1 (0.4)	0.7 (0.48)	-
3s	3, 10, 4	0.3 (0.08)	0.9 (0.27)	1.33 (0.13)
4s	9, 9, 9	0.51 (0.4)	0.46 (0.21)	0.59 (0.3)
5s	9, 9, -	0.56 (0.07)	0.7 (0.09)	-
6s	9, 8, -	0.82 (0.09)	0.95 (0.09)	-
7c	6, 7, -	0.7 (0.07)	0.81 (0.2)	-

Severe strain of ZYMV which found in Israel in the Jordan Valley (JV).

^ The engineered virus of ZYMV.

~ ZYMV weak strain described by Lecoq et al. (1991).

* Average of O.D (405) detected by ELISA from 11 plants.

** Standard deviation (in brackets).

+ s and c are squash and cucumber test plants, respectively.

Table 2. The stability of the ZYMV-AGI virus in the plants

plant species	number of tested plants: bombardment with 35SAG1	* visual symptoms: mild	* visual symptoms: severe	# molecular analysis of Ilu-180 mutation
squash	402	398	0	10
cucumber	105	103	0	5
melon	30	30	0	3
Total	537^	531+	0	18

*Visual symptoms were observed and detected by ELISA about one and half month post inoculation.

The presence of the Ilu Mutation was confirmed by digestion of the RT-PCR by Eco47III restriction enzyme.

^ Total of bombarded plants.

+ Total of infected plants

Table 3. Cross protection in squash with the mild strain ZYMV-AG1 (induction) against the severe strain ZYMV-JV (challenge) in the greenhouse experiments.

	Number of tested plants				
experiment number*	induction ZYMV-AG1	#challenge ZYMV-JV	symptoms mild	severe	~fruit damage
a)	47	47	45	2	1
a)	14	-	15		0
a)	-	5		5	5
b)	15	15	14	0	0
b)	5	-	5		0
b)	-	5		5	5
c)	43	field inocul.	43		0
c)	-	6		6	6
c)18 healthy	-	field inocul.		7	7

* a, b and c are three separate experiments. a and b were in the greenhouse and c was done in a small plot in the field. c is a sum of two experiments where the protected plants (AGI) were exposed to field inoculation.

~ No. of plants showed fruit damage.

Inoculation by aphids.

CLAIMS

1) A recombinant potyvirus infectious nucleic acid construct useful for plant cross protection, comprising a full length clone characterized in that its HC- Pro gene conserved FRNK box sequence contains a substitution.

2) A recombinant construct according to claim 1 wherein the nucleic acid is cDNA or an RNA transcript.

3) A recombinant construct according to claim 1 wherein the substitution in the conserved FRNK box is a substitution of Arg.

4) A recombinant construct according to claim 3 wherein Arg is substituted with an amino acid of the hydrophobic group or having a bulky side chain.

5) A recombinant construct according to claim 4 wherein Arg is substituted with Ile.

6) A recombinant construct according to claim 1 further containing a substitution which effectively abolishes aphid transmissibility.

7) A recombinant potyvirus infectious nucleic acid construct according to claim 5 and 6 wherein the potyvirus is ZYMV.

8) A recombinant construct according to claim 6 and 7 wherein the substitution which effectively abolishes aphid transmissibility is a substitution of the Ala residue at position 10 in the conserved DAG triplet in the N terminal region of the CP or substitution of Thr at position 309 in the HC - pro in ZYMV.

9) A recombinant construct according to claim 8 wherein the construct is ZYMV-AG1.

10) A recombinant construct according to claim 9, useful for plant cross protection wherein the cross protection is against severe strains of ZYMV.

11) A recombinant construct according to claims 1-10 further useful for the transient expression of foreign nucleic acid in plants wherein the full length clone has, in any position, a sequence of DNA or RNA inserted into it or wherein any viral genes are removed from the full length clone.

12) A recombinant potyvirus infectious nucleic acid construct according to claims 1-6 and claim 11 wherein the potyvirus is selected from BCMV, BYMV, BtMV, MWMV, OYDV, PRSV, PStV, PepMoV, PVMV, CGVBV, GEV, ISMV, JGMV, LYSV, LMV, MDMV, PPV, PVA, PVV , PVY, SCMV, SPFMV, TEV, TVMV, TBV, TuMV, WMV-2 , YMV and ZYFV.

13) A method for providing protection against viral infection in plants comprising inoculating plants with the recombinant potyvirus infectious construct as defined in any of the preceding claims.

14) A method according to claim 13 wherein inoculating plants is done by mechanical inoculation or by bombardment.

15) A method for introducing foreign nucleic acid into plants comprising infecting a plant with a full length clone as defined in claim 11 or co-infecting a plant with a full length clone as defined in claim 1, from which any viral genes are removed, together with a full length clone or virus harboring a full length clone.

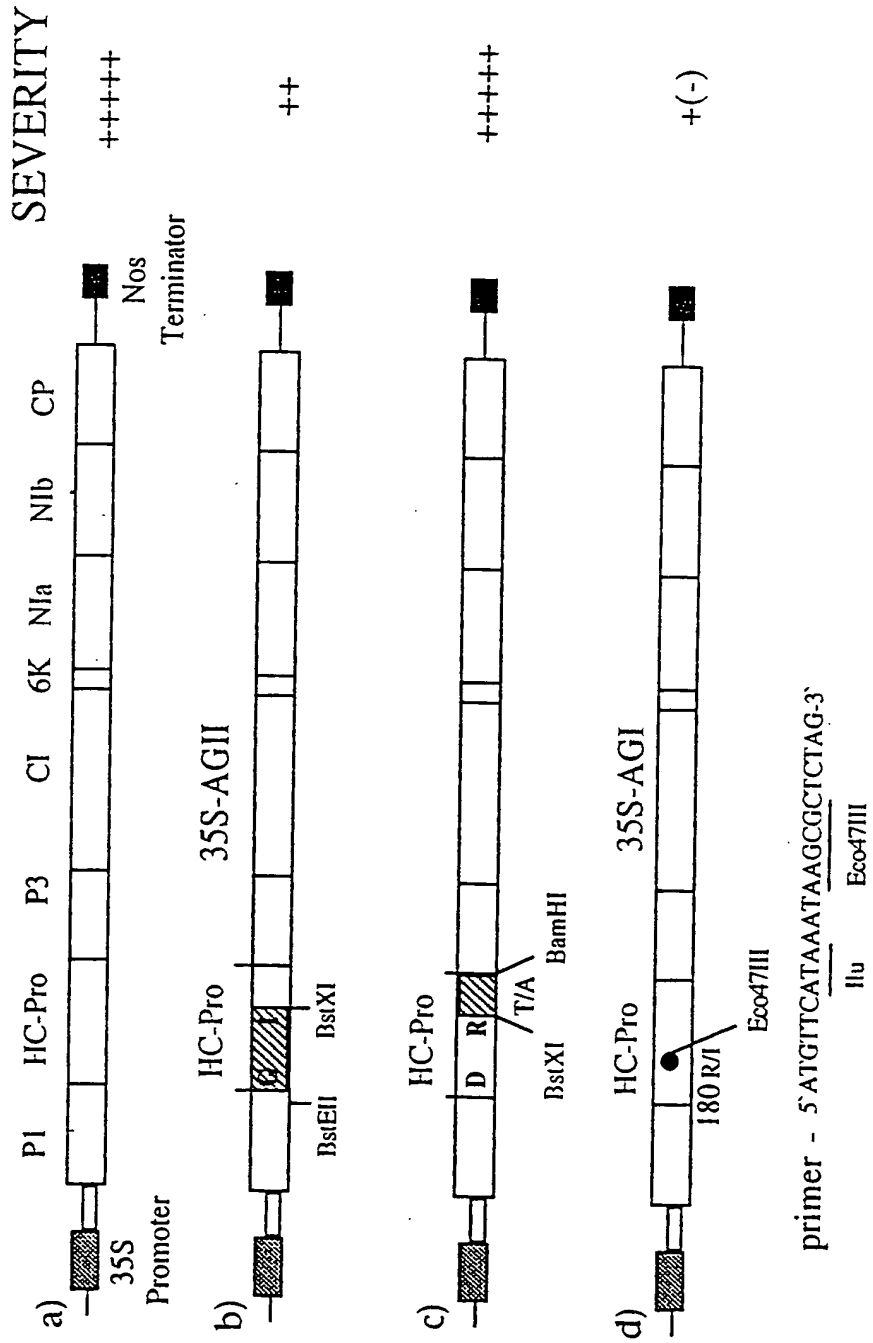
16) A method for the production of a mild strain of potyvirus comprising inoculating plants with the recombinant potyvirus infectious construct as defined in any of the preceding claims and collecting the resulting progeny.

17) A virus containing the recombinant construct as defined by claim 1 and as produced according to claim 16.

18) Produce inoculated with the recombinant construct as defined in the preceding claims and in the method according to claim 12.

19) Produce according to claim 18 wherein the produce are curcurbits.

20) Compositions for plant inoculation or for transient expression of foriegn nucleic acid in plants containing, as an active ingredient, the recombinant construct according to claim 1-12 or a virus containing the recombinant construct according to claim 17.


SEVERITY
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a)
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35S Promoter
P1
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NIa
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CP
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35S-AGII
BstEII
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FIGURE 1

CLAIMS

1) A recombinant potyvirus infectious nucleic acid construct useful for plant cross protection, comprising a full length clone characterized in that its HC- Pro gene conserved FRNK box sequence contains a substitution.

2) A recombinant construct according to claim 1 wherein the nucleic acid is cDNA or an RNA transcript.

3) A recombinant construct according to claim 1 wherein the substitution in the conserved FRNK box is a substitution of Arg.

4) A recombinant construct according to claim 3 wherein Arg is substituted with an amino acid of the hydrophobic group or having a bulky side chain.

5) A recombinant construct according to claim 4 wherein Arg is substituted with Ile.

6) A recombinant construct according to claim 1 further containing a substitution which effectively abolishes aphid transmissibility.

7) A recombinant potyvirus infectious nucleic acid construct according to claim 5 and 6 wherein the potyvirus is ZYMV.

8) A recombinant construct according to claim 6 and 7 wherein the substitution which effectively abolishes aphid transmissibility is a substitution of the Ala residue at position 10 in the conserved DAG triplet in the N terminal region of the CP or substitution of Thr at position 309 in the HC - pro in ZYMV.

9) A recombinant construct according to claim 8 wherein the construct is ZYMV-AG1.

10) A recombinant construct according to claim 9, useful for plant cross protection wherein the cross protection is against severe strains of ZYMV.

11) A recombinant construct according to claims 1-10 further useful for the transient expression of foreign nucleic acid in plants wherein the full length clone has, in any position, a sequence of DNA or RNA inserted into it or wherein any viral genes are removed from the full length clone.

12) A recombinant potyvirus infectious nucleic acid construct according to claims 1-6 and claim 11 wherein the potyvirus is selected from BCMV, BYMV, BtMV, MWMV, OYDV, PRSV, PStV, PepMoV, PVMV, CGVBV, GEV, ISMV, JGMV, LYSV, LMV, MDMV, PPV, PVA, PVV , PVY, SCMV, SPFMV, TEV, TVMV, TBV, TuMV, WMV-2 , YMV and ZYFV.

13) A method for providing protection against viral infection in plants comprising inoculating plants with the recombinant potyvirus infectious construct as defined in any of the preceding claims.

14) A method according to claim 13 wherein inoculating plants is done by mechanical inoculation or by bombardment.

15) A method for introducing foreign nucleic acid into plants comprising infecting a plant with a full length clone as defined in claim 11 or co-infecting a plant with a full length clone as defined in claim 1, from which any viral genes are removed, together with a full length clone or virus harboring a full length clone.

16) A method for the production of a mild strain of potyvirus comprising inoculating plants with the recombinant potyvirus infectious construct as defined in any of the preceding claims and collecting the resulting progeny.

17) A virus containing the recombinant construct as defined by claim 1 and as produced according to claim 16.

18) Produce inoculated with the recombinant construct as defined in the preceding claims and in the method according to claim 12.

19) Produce according to claim 18 wherein the produce are curcurbits.

20) Compositions for plant inoculation or for transient expression of foriegn nucleic acid in plants containing, as an active ingredient, the recombinant construct according to claim 1-12 or a virus containing the recombinant construct according to claim 17.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/40, 15/57, 15/82, 15/83, 7/04, 7/00, A01N 63/02

A3

(11) International Publication Number: WO 99/51749

(43) International Publication Date: 14 October 1999 (14.10.99)

(21) International Application Number: PCT/IL99/00184

(22) International Filing Date: 30 March 1999 (30.03.99)

(30) Priority Data:
123994 7 April 1998 (07.04.98) IL

(71) Applicant *(for all designated States except US)*: STATE OF ISRAEL/MINISTRY OF AGRICULTURE [IL/IL]; Agricultural Research Organization, The Volkani Center, 50250 Bet Dagan (IL).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: GAL-ON, Amit [IL/IL]; Vitkin Street 16, 47295 Ramat Hasharon (IL).

(74) Agent: NOAM, Meir; P.O. Box 34335, 91342 Jerusalem (IL).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
9 December 1999 (09.12.99)

(54) Title: RECOMBINANT POTYVIRUS CONSTRUCT AND USE THEREOF

(57) Abstract

The present invention relates to a recombinant potyvirus infectious nucleic acid, preferably cDNA or RNA, construct useful for plant cross protection, comprising a full length clone characterized in that its HC-Pro gene conserved FRNK box sequence contains a substitution, preferably a substitution of Arg. Preferably, the construct further contains a substitution which effectively abolishes aphid transmissibility. The present invention further relates to a method for the production of a mild strain of potyvirus utilizing the above mentioned construct and to a method for protecting plants from viral infection and for transient expression of foreign nucleic acid (genes) in plants, using said construct. Preferably, the present invention relates to a method for cross protection of curcurbits against ZYMV infection. The present invention also relates to a method for providing protection against viral infection in plants comprising inoculating plants, by mechanical inoculation or by bombardment, with the recombinant potyvirus infectious construct.

INTERNATIONAL SEARCH REPORT

Application No
PCT/IL 99/00184

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/40 C12N15/57 C12N15/82 C12N15/83 C12N7/04
C12N7/00 A01N63/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUET, H., ET AL: "Mutations in the helper component protease gene of zucchini yellow mosaic virus affect its ability to mediate aphid transmissibility" JOURNAL OF GENERAL VIROLOGY, vol. 75, 1994, pages 1407-1414, XP002118196 cited in the application the whole document	1-11, 17-20
Y	-& HUET, H., ET AL.: "Zucchini yellow mosaic virus (ZYMV) HC-PRO gene" EMBL ACCESSION NO: X77756, 25 April 1994 (1994-04-25), XP002118197 the whole document	8,12-16

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

' Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 October 1999	27/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GAL-ON, A., ET AL.: "A zucchini yellow mosaic virus coat protein gene mutation restores aphid transmissibility but has no effect on amplification" JOURNAL OF GENERAL VIROLOGY, vol. 73, 1992, pages 2183-2187, XP002118198 cited in the application the whole document	8
Y	MAIA, I.G., ET AL.: "Potyviral HC-PRO: a multifunctional protein" JOURNAL OF GENERAL VIROLOGY, vol. 77, 1996, pages 1335-1341, XP002118199	12
A	the whole document	1-11, 13-20
Y	FUCHS, M., ET AL.: "Management of virus diseases by classical and engineered protection" MOLECULAR PLANT PATHOLOGY ON-LINE 'HTTP://WWW.BSPP.ORG.UK/MPPOL/! 1997/0116FUCHS, 'Online! 1997, pages 1-19, XP002118200 Retrieved from the Internet: <URL:http://www.bspp.org.uk./mppol/1997/0116fuchs/> 'retrieved on 1999-10-08! see section 2.3 pages 5-6	13,14
Y	WO 95 12669 A (TEXAS A & M UNIVERSITY SYST) 11 May 1995 (1995-05-11) the whole document	15
Y	LECOQ, H., ET AL.: "Control of zucchini yellow mosaic virus in squash by cross protection" PLANT DISEASE, vol. 75, 1991, pages 208-211, XP002118201 the whole document	16

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GRANIER, F., ET AL.: "Mutations in zucchini yellow mosaic virus helper component protein associated with loss of aphid transmissibility" JOURNAL OF GENERAL VIROLOGY, vol. 74, 1993, pages 2737-2742, XP002118202 the whole document -& GRANIER, F., ET AL.: "Helper component - zucchini yellow mosaic virus (strain E15-PAT)" PIR ACCESSION NO:JQ2396, 30 September 1993 (1993-09-30), XP002118203 the whole document	1-20
A	LECOQ, H., ET AL.: "Characterization of a zucchini yellow mosaic virus isolate with a deficient helper component" PHYTOPATHOLOGY, vol. 81, 1991, pages 1087-1091, XP002118204 the whole document	1-20
A	PENG, Y.-H., ET AL.: "Mutations in the HC-Pro gene of zucchini yellow mosaic potyvirus: effects on aphid transmission and binding to purified virions" JOURNAL OF GENERAL VIROLOGY, vol. 79, April 1998 (1998-04), pages 897-904, XP002118215 the whole document	1-20
A	BLANC, S., ET AL.: "A specific interaction between coat protein and helper component correlates with aphid transmission of a potyvirus" VIROLOGY, vol. 231, 1997, pages 141-147, XP002118205 the whole document	8
A	LIVNEH, ORNA., ET AL.: "Plants transformed with the first (nonstructural) three cistrons of potato virus Y are resistant to potato virus infection" TRANSGENICS (1995), 1(5), 565-71, XP002118206 the whole document	13

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GAL-ON, A., ET AL.: "Particle bombardment drastically increases the infectivity of cloned DNA of zucchini yellow mosaic potyvirus" JOURNAL OF GENERAL VIROLOGY, vol. 76, 1995, pages 3223-3227, XP002118207 the whole document	14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IL 99/00184

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9512669 A	11-05-1995	US 5491076 A	13-02-1996
		AU 1408095 A	23-05-1995
		US 5766885 A	16-06-1998
		ZA 9408561 A	30-06-1995

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
NOAM, Meir
Attn. DR.MEIR NOAM.
P.O. Box 34335
Jerusalem 91342
ISRAEL

Date of mailing *(day/month/year)*	27/10/1999
Applicant's or agent's file reference: a645-49-V	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No.: PCT/IL 99/00184	International filing date *(day/month/year)* 30/03/1999
Applicant: STATE OF ISRAEL/MINISTRY OF AGRICULTURE et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Mireille Claudepierre

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

TENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
a645-49-V		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IL 99/00184	30/03/1999	07/04/1998
Applicant		
STATE OF ISRAEL/MINISTRY OF AGRICULTURE et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. –

   [ ] None of the figures.

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

INTERNATIONAL SEARCH REPORT

International Application No
IL 99/00184

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/40 C12N15/57 C12N15/82 C12N15/83 C12N7/04
C12N7/00 A01N63/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ³	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUET, H., ET AL: "Mutations in the helper component protease gene of zucchini yellow mosaic virus affect its ability to mediate aphid transmissibility" JOURNAL OF GENERAL VIROLOGY, vol. 75, 1994, pages 1407-1414, XP002118196 cited in the application the whole document	1-11, 17-20
Y	-& HUET, H., ET AL.: "Zucchini yellow mosaic virus (ZYMV) HC-PRO gene" EMBL ACCESSION NO: X77756, 25 April 1994 (1994-04-25), XP002118197 the whole document	8,12-16

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

³ Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 October 1999	27/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category ³	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GAL-ON, A., ET AL.: "A zucchini yellow mosaic virus coat protein gene mutation restores aphid transmissibility but has no effect on amplification" JOURNAL OF GENERAL VIROLOGY, vol. 73, 1992, pages 2183-2187, XP002118198 cited in the application the whole document	8
Y	MAIA, I.G., ET AL.: "Potyviral HC-PRO: a multifunctional protein" JOURNAL OF GENERAL VIROLOGY, vol. 77, 1996, pages 1335-1341, XP002118199	12
A	the whole document	1-11, 13-20
Y	FUCHS, M., ET AL.: "Management of virus diseases by classical and engineered protection" MOLECULAR PLANT PATHOLOGY ON-LINE 'HTTP://WWW.BSPP.ORG.UK/MPPOL/! 1997/0116FUCHS, 'Online! 1997, pages 1-19, XP002118200 Retrieved from the Internet: <URL:http://www.bspp.org.uk./mppol/1997/0116fuchs/> 'retrieved on 1999-10-08! see section 2.3 pages 5-6	13,14
Y	WO 95 12669 A (TEXAS A & M UNIVERSITY SYST) 11 May 1995 (1995-05-11) the whole document	15
Y	LECOQ, H., ET AL.: "Control of zucchini yellow mosaic virus in squash by cross protection" PLANT DISEASE, vol. 75, 1991, pages 208-211, XP002118201 the whole document	16

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category ³	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GRANIER, F., ET AL.: "Mutations in zucchini yellow mosaic virus helper component protein associated with loss of aphid transmissibility" JOURNAL OF GENERAL VIROLOGY, vol. 74, 1993, pages 2737-2742, XP002118202 the whole document -& GRANIER, F., ET AL.: "Helper component - zucchini yellow mosaic virus (strain E15-PAT)" PIR ACCESSION NO:JQ2396, 30 September 1993 (1993-09-30), XP002118203 the whole document	1-20
A	LECOQ, H., ET AL.: "Characterization of a zucchini yellow mosaic virus isolate with a deficient helper component" PHYTOPATHOLOGY, vol. 81, 1991, pages 1087-1091, XP002118204 the whole document	1-20
A	PENG, Y.-H., ET AL.: "Mutations in the HC-Pro gene of zucchini yellow mosaic potyvirus: effects on aphid transmission and binding to purified virions" JOURNAL OF GENERAL VIROLOGY, vol. 79, April 1998 (1998-04), pages 897-904, XP002118215 the whole document	1-20
A	BLANC, S., ET AL.: "A specific interaction between coat protein and helper component correlates with aphid transmission of a potyvirus" VIROLOGY, vol. 231, 1997, pages 141-147, XP002118205 the whole document	8
A	LIVNEH, ORNA., ET AL.: "Plants transformed with the first (nonstructural) three cistrons of potato virus Y are resistant to potato virus infection" TRANSGENICS (1995), 1(5), 565-71, XP002118206 the whole document	13

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GAL-ON, A., ET AL.: "Particle bombardment drastically increases the infectivity of cloned DNA of zucchini yellow mosaic potyvirus" JOURNAL OF GENERAL VIROLOGY, vol. 76, 1995, pages 3223-3227, XP002118207 the whole document	14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
/IL 99/00184

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9512669 A	11-05-1995	US 5491076 A	13-02-1996
		AU 1408095 A	23-05-1995
		US 5766885 A	16-06-1998
		ZA 9408561 A	30-06-1995

16

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
a645-49-V		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IL99/00184	30/03/1999	07/04/1998
International Patent Classification (IPC) or national classification and IPC C12N15/40		
Applicant STATE OF ISRAEL/MINISTRY OF AGRICULTURE et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
28/10/1999	11.07.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Petri, B Telephone No. +49 89 2399 7356

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL99/00184

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-16 as originally filed

Claims, No.:

1-20 as received on 01/06/2000 with letter of 01/06/2000

Drawings, sheets:

1/1 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

see separate sheet

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to nov lty, inventive st p or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 7-11, 15
	No:	Claims 1-6, 12-14, 16-20
Inventive step (IS)	Yes:	Claims 7
	No:	Claims 8-11, 15
Industrial applicability (IA)	Yes:	Claims 1-20
	No:	Claims none

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: Huet et al. 1994 J. Gen. Virol. 75:1407-1414; XP-002 118 196;
D2: Embl. Accession X77756; XP-002 118 197;
D3: Lecoq et al. 1991 Plant Disease 75:208-211; XP-002 118 201;
D4: Gal On et al. 1992 J. Gen. Virol. 73:2183-2187; XP-002 118 198;
D5: Granier et al. 1993 J. Gen. Virol. 74:2737-2742; XP-002 118 202;
D6: WO 95/12669;

Novelty; Art 33(2), PCT

1) The subject-matter of claims 1-6, 12-14, 16-20 is not novel (Article 33(2) PCT).

1.1) D1 discloses recombinant potyvirus infectious nucleic acid constructs, comprising a full length clone (pZYMK-HC(GI-T)) characterized in that its HC-Pro gene conserved FRNK box sequence contains a substitution (pZYMK-HC(GI-T) (Fig. 1b, last bar).

Irrespective of the question whether pZYMK-HC(GI-T) were known to be useful in plant cross protection, said feature is inherent to above hybrid strain pZYMK-HC(GI-T) as its HC-Pro gene comprises a substitution in the FRNK box, i.e. Arg^{180} to Ile^{180}, which, as has been shown by the applicant, confers usefulness in plant cross protection.

In addition, the wording *"characterized only"* does not exclude that the full-length clone contains in addition substitutions at any other position of the virus.

Consequently subject-matter of claim 1 lacks novelty in view of the full length clone pZYMK-HC(GI-T) of D1.

1.2) As the full length clone pZYMK-HC(GI-T) of D1 comprises inserted sequences of DNA or RNA (e.g. the T7 promotor) the constructs of claim 12 also lack novelty.

1.3) As the full length clone pZYMK-HC(GI-T) of D1 is used for inoculating plants and to obtain progeny viruses, the methods of claim 13-14 and 16, the virus of claim 17 the produce of claim 18-19 and the composition of claim 20 also is considered to lack novelty in view of D1.

2.) Subject-matter of claim 7-11, 15 is considered novel.

2.1) As the HC-Pro sequence of full length clone of D1 differs from the sequence of ZYMV-AG1 of present claim 1 at position 148 (pZYMK-HC(GI-T):Gly148 vs. ZYMV-AG1:Asp148) subject-matter of claim 7 is considered to be novel.

2.2) Although strain ZYMK-HC(-) comprises a further mutation which effectively abolishes aphid transmissibility (i.e. the mutation at pos 308, see D1 Table 2) subject-matter of claim 8 is considered novel, as apparently isolate of strain ZYMK-HC(-) was not available as infectious nucleic acid full length clone.

2.3) Although the mutation at position 10 of the DAG triplet of the CP locus and its effect on aphid transmissibility is known from D4, subject-matter of claim 9 is considered novel as neither D1 nor D4 show infectious full length clones of potyvirus which comprise both mutations.

2.4) As subject-matter of claim 7 is novel, subject-matter of dependent claim 10 is also considered novel.

2.5) As D1 comprises only isolates and full length clones of ZYMV, subject-matter of claims 11 and 15, which is restricted to different potyviruses, is not disclosed by D1 and thus considered novel.

Inventive Step; Art. 33(3), PCT

3.) The subject-matter proposed in claims 8-11, 15 of the present application cannot be considered as involving an inventive step (Article 33(3) PCT) for the following reasons:

3.1) Inclusion of the substitution of the Ala^{10} in the conserved DAG triplet in the N terminal

region of CP into the context of the present application lacks an inventive step, since its effect, namely the cause of loss of aphid transmissibility is known from D4. As the technical effect of said additional substitution is restricted to the known phenotype caused by that substitution, i.e. said loss of aphid transmissibility, it would be obvious to the person skilled in the art to include this feature into any strain ZYMK, in order to obtain aphid intransmissibility (see also PCT Gazette IV-8.8 A1 (iii)).
Consequently subject-matter of claims 8-10 is considered not to be based on an inventive step

3.2) Given the high degree of similarity among members of the potyvirus group, the extrapolation of the known subject-matter of claim 1-6 (see item 1) to other such closely related viruses is not considered to involve an inventive step. As such claim 11, 15 are considered not to comprise an inventive contribution to the art.

4) Subject-matter of claim 7 and any subject-matter dependent thereof is considered to involve an inventive step.

D1 is considered to represent the closest prior art, and discloses an infectious nucleic acid full length clone of a potyvirus i.e. pZYMV-HC(GI-T), characterized in that the amino acid residue of the HC-Pro gene at position 148 is Gly and at position 180 is Ile. The subject-matter of present claim 7 differs from said closest prior art in that a different full length clone of potyvirus is provided i.e. ZYMV-AG1 with Asp at position 148 and Ile at position 180 of said gene.

The technical effect of said difference is that a strain is provided which is *"useful in cross protection"*.

As such a technical effect is already known from D3 and is inherent to pZYMV-HC(GI-T) of D1, the technical problem to be solved may be considered as to provide alternative strains which are *"useful in plant cross protection"*.

As none of the available prior art disclosures indicated a link between the *"usefulness in plant cross protection"* and mutations in the FNRK box of the HC-Pro locus, the specific construct as depicted in Fig. 1 d) is considered inventive.

Re Item VIII

Certain observations on the international application

5. Claim 7 does not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. Construct/strain denomination ZYMV-AG1 is arbitrary and thus meaningless to the skilled person. The technical features characterising said construct/strain need to be included into the claim (see however below).

   In said context it is to be noted that although said strain may be characterized in the description, in order to fulfill the requirements of Art. 6, the claim itself has to contain all the necessary technical features characterizing the subject-matter for which protection is thought.

6. The above lack of novelty (item 1), seems mainly to be due to the wording of claim 1:

   The Examining Division recognizes the applicants finding that out of the three known mutations in the HC-Pro gene of the mild and poorly aphid transmissible strain ZYMK-WK as disclosed in D1 (i.e. Asp148 to Gly148, Arg180 to Ile180, Thr308 to Ala308) a single substitution, i.e. Ile180, is sufficient to cause the "mild" phenotype and to render strains of potyvirus "usefull in plant cross protection".

   However, the scope of present claim 1 comprises any potivirus full length clone that shows a substitution in the FRNK motiv of the HC-Pro locus (as neither the wording *characterized in*, nor *characterized* ***only*** *in*, excludes additional features such as amino acid variation at other positions). In view pZYMV-HC(GI-T) of D1 any claim to a full length clone not restricted to such clones which do not exhibit the other mutations comprised in HC-Pro of pZYMV-HC(GI-T) of D1 lacks novelty.

   In any case, the general term *"substitution"* without a reference sequence does not pose any limitation to the scope. This seems particularly true for viral genes where several isolates with varying sequences are known (see D1 Fig. 2) with no apparent wild type sequence.

CLAIMS

1) A recombinant potyvirus infectious nucleic acid construct useful for plant cross protection, comprising a full length clone characterized only in that its HC- Pro gene conserved FRNK box sequence contains a substitution.

2) A recombinant construct according to claim 1 wherein the nucleic acid is cDNA or an RNA transcript.

3) A recombinant construct according to claim 1 wherein the substitution in the conserved FRNK box is a substitution of Arg.

4) A recombinant construct according to claim 3 wherein Arg is substituted with an amino acid of the hydrophobic group or having a bulky side chain.

5) A recombinant construct according to claim 4 wherein Arg is substituted with Ile.

6) A recombinant potyvirus infectious nucleic acid construct according to claim 1-5 wherein the potyvirus is ZYMV.

7) A recombinant construct according to claim 6 wherein the construct is ZYMV-AG1.

8) A recombinant construct according to claim 1-7 further containing a substitution which effectively abolishes aphid transmissibility.

9) A recombinant construct according to claim 8 wherein the substitution which effectively abolishes aphid transmissibility is a substitution of the Ala residue at position 10 in the conserved DAG triplet in the N terminal region of the CP.

AMENDED SHEET

10) A recombinant construct according to claim 7,8 and 9 useful for plant cross protection wherein the cross protection is against severe strains of ZYMV.

11) A recombinant potyvirus infectious nucleic acid construct according to claims 1-6 wherein the potyvirus is selected from BCMV, BYMV, BtMV, MWMV, OYDV, PRSV, PStV, PepMoV, PVMV, CGVBV, GEV, ISMV, JGMV, LYSV, LMV, MDMV, PPV, PVA, PVV , PVY, SCMV, SPFMV, TEV, TVMV, TBV, TuMV, WMV-2 , YMV and ZYFV.

12) A recombinant construct according to claims 1-11 further useful for the transient expression of foreign nucleic acid in plants wherein the full length clone has, in any position, a sequence of DNA or RNA inserted into the full length clone.

13) A method for providing protection against viral infection in plants comprising inoculating plants with the recombinant potyvirus infectious construct as defined in any of the preceding claims.

14) A method according to claim 13 wherein inoculating plants is done by mechanical inoculation or by bombardment.

15) A method for introducing foreign nucleic acid into plants comprising infecting a plant with a full length clone as defined in claim 11.

16) A method for the production of a mild strain of potyvirus comprising inoculating plants with the recombinant potyvirus infectious construct as defined in any of the preceding claims and collecting the resulting progeny.

AMENDED SHEET

17) A virus containing the recombinant construct as defined by claim 1 and as produced according to claim 16.

18) Produce inoculated with the recombinant construct as defined in the preceding claims and in the method according to claim 13.

19) Produce according to claim 18 wherein the produce are curcurbits.

20) Compositions for plant inoculation or for transient expression of foreign nucleic acid in plants containing, as an active ingredient, the recombinant construct according to claim 1-12 or a virus containing the recombinant construct according to claim 17.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
a645-49-V		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IL 99/00184	30/03/1999	07/04/1998

Applicant

STATE OF ISRAEL/MINISTRY OF AGRICULTURE et al.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. –

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]IL 99/00184

A. CLASSIFICATION OF SUBJECT MAT[redacted]
IPC 6 C12N15/40 C12N15/57 C12N15/82 C12N15/83 C12N7/04
C12N7/00 A01N63/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUET, H., ET AL: "Mutations in the helper component protease gene of zucchini yellow mosaic virus affect its ability to mediate aphid transmissibility" JOURNAL OF GENERAL VIROLOGY, vol. 75, 1994, pages 1407-1414, XP002118196 cited in the application	1-11, 17-20
Y	the whole document -& HUET, H., ET AL.: "Zucchini yellow mosaic virus (ZYMV) HC-PRO gene" EMBL ACCESSION NO: X77756, 25 April 1994 (1994-04-25), XP002118197 the whole document	8,12-16
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 October 1999	27/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Maddox, A

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GAL-ON, A., ET AL.: "A zucchini yellow mosaic virus coat protein gene mutation restores aphid transmissibility but has no effect on amplification" JOURNAL OF GENERAL VIROLOGY, vol. 73, 1992, pages 2183-2187, XP002118198 cited in the application the whole document	8
Y	MAIA, I.G., ET AL.: "Potyviral HC-PRO: a multifunctional protein" JOURNAL OF GENERAL VIROLOGY, vol. 77, 1996, pages 1335-1341, XP002118199	12
A	the whole document	1-11, 13-20
Y	FUCHS, M., ET AL.: "Management of virus diseases by classical and engineered protection" MOLECULAR PLANT PATHOLOGY ON-LINE 'HTTP://WWW.BSPP.ORG.UK/MPPOL/! 1997/0116FUCHS, 'Online! 1997, pages 1-19, XP002118200 Retrieved from the Internet: <URL:http://www.bspp.org.uk./mppol/1997/0116fuchs/> 'retrieved on 1999-10-08! see section 2.3 pages 5-6	13,14
Y	WO 95 12669 A (TEXAS A & M UNIVERSITY SYST) 11 May 1995 (1995-05-11) the whole document	15
Y	LECOQ, H., ET AL.: "Control of zucchini yellow mosaic virus in squash by cross protection" PLANT DISEASE, vol. 75, 1991, pages 208-211, XP002118201 the whole document	16

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	GRANIER, F., ET AL.: "Mutations in zucchini yellow mosaic virus helper component protein associated with loss of aphid transmissibility" JOURNAL OF GENERAL VIROLOGY, vol. 74, 1993, pages 2737-2742, XP002118202 the whole document -& GRANIER, F., ET AL.: "Helper component - zucchini yellow mosaic virus (strain E15-PAT)" PIR ACCESSION NO:JQ2396, 30 September 1993 (1993-09-30), XP002118203 the whole document	1-20
A	LECOQ, H., ET AL.: "Characterization of a zucchini yellow mosaic virus isolate with a deficient helper component" PHYTOPATHOLOGY, vol. 81, 1991, pages 1087-1091, XP002118204 the whole document	1-20
A	PENG, Y.-H., ET AL.: "Mutations in the HC-Pro gene of zucchini yellow mosaic potyvirus: effects on aphid transmission and binding to purified virions" JOURNAL OF GENERAL VIROLOGY, vol. 79, April 1998 (1998-04), pages 897-904, XP002118215 the whole document	1-20
A	BLANC, S., ET AL.: "A specific interaction between coat protein and helper component correlates with aphid transmission of a potyvirus" VIROLOGY, vol. 231, 1997, pages 141-147, XP002118205 the whole document	8
A	LIVNEH, ORNA., ET AL.: "Plants transformed with the first (nonstructural) three cistrons of potato virus Y are resistant to potato virus infection" TRANSGENICS (1995), 1(5), 565-71, XP002118206 the whole document	13

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INTERNATIONAL SEARCH REPORT

International Application No
[redacted]IL 99/00184

C.(Continuation) DOCUMENTS CONSIDE[redacted]O BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	GAL-ON, A., ET AL.: "Particle bombardment drastically increases the infectivity of cloned DNA of zucchini yellow mosaic potyvirus" JOURNAL OF GENERAL VIROLOGY, vol. 76, 1995, pages 3223-3227, XP002118207 the whole document	14

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
IL 99/00184

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9512669 A	11-05-1995	US 5491076 A	13-02-1996
		AU 1408095 A	23-05-1995
		US 5766885 A	16-06-1998
		ZA 9408561 A	30-06-1995

RECOMBINANT POTYVIRUS CONSTRUCT AND USE THEREOF

Field of the Invention

The present invention generally relates to a recombinant potyvirus infectious nucleic acid construct useful for providing protection against viral infection in plants and to a recombinant virus harboring said construct. More specifically, the present invention relates to a recombinant potyvirus infectious construct containing an HC - Pro gene whose sequence coding for the conserved FRNK box contains a substitution. Preferably, the Arginin (Arg) is substituted with Isoleucine (Ile).

The present invention further relates to a method for the production of a mild strain of potyvirus utilizing the above mentioned construct and to a method for protecting plants from viral infection and for transient expression of foreign nucleic acid (genes) in plants, using said construct. Preferably, the present invention relates to a method for cross protection of curcurbits against ZYMV infection.

Background of the Invention

The Curcurbitaceae is a broad botanical family comprising several economically important species cultivated worldwide, such as cucumber, squash, cantaloupe, zucchini pumpkin, melon and watermelon. Curcurbit production throughout the world is impaired by several aphid transmitted viruses, the most prevalent being the two potyviruses ZYMV (Zucchini Yellow Mosaic Virus) and WMV-2 (Watermelon Mosaic Virus 2) and CMV (cucumber Mosaic Virus). ZYMV infected plants show symptoms such as vein clearing followed by a yellow mosaic on the infected systemic leaf and may show stunting and distortion. In mild cases of infection the quantity and quality of the yield are damaged and in severe infections there might be a total loss of the yield, causing significant economical losses.

Control measures include phytosanitation, the use of colored plastic mulches for attracting virus bearing aphids and creating a hydrophobic barrier around the plant such as oil sprays. These provide temporary protection and are a limited protection during a massive infection.

Development of virus resistant cultivars either by classical breeding or by introducing viral derived nucleic acid sequences into the plant genome through genetic engineering of plants, is also employed for the protection of plants against virus infection. Squash hybrid transgenic inbred lines exhibiting resistance to ZYMV were produced (Tricoli D.M., Carney K.J., Russell McMaster P.F., Groff D.W., Hadden K.C., Himmel P.T., Hubbard J. P., Boeshore M.L. and Quemada H.D. (1995) *Biotechnology* vol. 13;1458) but these are limited to one cultivar only.

The phenomenon of cross protection, which is the use of a mild strain of a virus to protect against the damage by infection with severe strains of the same virus, provides a good method for controlling virus diseases.

In curcurbits, cross protection, specifically against ZYMV, is an attractive control option. Cross protection is highly effective under severe disease pressure. The severity of the disease conferred by the ZYMV on curcurbits and the latter's relatively short crop cycle (8 - 16 weeks) make cross protection a preferred control option for curcurbits.

The currently used mild strain of ZYMV for cross protection of curcurbits, was obtained by Lecoq (Lecoq H., Lemaire JM., Wipf-Scheible C., (1991) *Plant Dis.* 75:208-211). This strain is designated ZYMV-WK and is poorly transmitted by aphids, causes only mild leaf mottling and does not induce fruit malformation in curcurbits. Plants are inoculated at an early stage with the mild strain (ZYMV-WK), usually by mechanical inoculation.

No full length infectious clone of this mild virus exists.

Potyviruses have a genome consisting of a positive - sense single stranded RNA possessing a covalently linked 5' - terminal viral protein (Vpg) and a 3'

terminal poly (A) tail. The viral RNA is expressed as a single polyprotein, which is subsequently processed by three virus encoded proteases, producing eight to ten genes, which are a conserved region throughout the potyvirus genome. The potyviruses are transmitted from plant to plant by aphids in a non persistent manner, and this process is dependent on the presence of two virus encoded proteins, the coat protein (CP) and the helper component proteinase HC-Pro. The HC-Pro is a multifunctional protein involved in aphid transmission, polyprotein processing, virus replication, symptom expression and in virus movement in the plant (Maia I. G., Haenni A., and Bernardi F., (1996) *Journal of General Virology* 77:1335-1341).

Zucchini yellow mosaic virus (ZYMV) is a member of the potyvirus group which causes devastating epidemics in commercial curcurbits world wide. A full length clone of ZYMV, from which infectious transcripts were produced, was constructed (Gal On A., Antignus Y., Rosner A., and Raccah B. (1991) *Journal of General Virology* 72:2639-2643).

It was found that a substitution of the Alanin (Ala) residue to Threonin (Thr) at position 10 in the conserved DAG (Aspartate - Alanin - Glycine; Asp-Ala-Gly) triplet in the N terminal region of the CP effectively abolished aphid transmissibility of ZYMV (Gal On A., Antignus Y., Rosner A., and Raccah B. (1992) *Journal of General Virology* 73:2183-2187). Also substitution of Thr by Ala at position 309 in the HC-Pro gene of the infectious clone of ZYMV effected aphid transmissibility without changing virus accumulation and symptom development (Huet H., Gal On A., Meiri E., Lecoq H. and Raccah B.(1994) *Journal of General Virology* 75:1407-1414), though less effectively than the substitution in the DAG triplet in the CP of the ZYMV.

It has surprisingly been found that an amino acid substitution in the conserved FRNK box of the potyvirus HC-pro gene allows for the construction of an infectious potyvirus construct, which, when introduced to plants, induces little or no symptom development, and which does not effect the accumulation of the

virus in the plant. This infectious construct is therefore a unique potyvirus construct which is highly superior for plant cross protection and for transient expression of foreign nucleic acid in plants. It has an improved ability of protection against infection by the severe strain of ZYMV, over any of the existing protection methods, is significantly safer and more environment friendly than the naturally occurring viruses, does not cause the development of symptoms in a variety of curcurbits, and is stable (no revertant virus has been found after several passages through plants).

Summary of the Invention

The present invention relates to a recombinant potyvirus infectious nucleic acid, preferably cDNA or RNA, construct useful for plant cross protection, comprising a full length clone characterized in that its HC- Pro gene conserved FRNK box sequence contains a substitution, preferably a substitution of Arg.

Preferably, the construct further contains a substitution which effectively abolishes aphid transmissibility, such as a substitution of the Ala residue at position 10 in the conserved DAG triplet in the N terminal region of the CP or substitution of Thr at position 309 in the HC - pro in ZYMV.

The recombinant construct of the present invention may be useful for plant cross protection (especially against severe strains of ZYMV) and for the transient expression of foreign nucleic acid in plants wherein the full length clone has, in any position, a sequence of DNA or RNA inserted into it or wherein any viral genes are removed from the full length clone (defective RNA). The full length clone may be of any potyvirus, preferably of ZYMV.

The present invention also relates to a method for providing protection against viral infection in plants comprising inoculating plants, by mechanical inoculation or by bombardment, with the recombinant potyvirus infectious construct.

The present invention also relates to a method for introducing foreign nucleic acid into plants according comprising infecting a plant with a full length clone or co-infecting a plant with a full length clone, from which any viral genes are removed, together with a full length clone or virus harboring a full length clone.

The present invention also relates to a method for the production of a mild strain of potyvirus comprising inoculating plants with the recombinant potyvirus infectious construct as defined in any of the preceding claims and collecting the resulting progeny, and to a virus produced in this method.

The present invention further relates to compositions for plant inoculation or for transient expression of foriegn nucleic acid in plants containing, as an active ingredient, the recombinant construct or a virus containing the recombinant construct.

Detailed Description of the Invention

The present invention relates to a recombinant potyvirus infectious nucleic acid construct useful for plant cross protection and for the transient expression of foreign nucleic acid in plants. The present invention further relates to compositions for plant inoculation or for transient expression of foriegn nucleic acid in plants containing, as an active ingredient, the recombinant construct or a virus containing the recombinant construct.

The construct of the present invention comprises a full length potyvirus clone containing a substitution in the conserved FRNK box sequence in the HC - pro gene, preferably, Arg (in the FRNK box) is substituted with an amino acid having a bulky side chain or an amino acid from the hydrophobic group such as Ile. This substitution in the FRNK box dramatically effects the severity of symptom development without effecting the accumulation of the virus in the plant. Preferably, the construct of the present invention also contains a substitution which effectively abolishes aphid transmissibility , such as the

substitution of the Ala residue to Thr at position 10 in the conserved DAG (Asp-Ala-Gly) triplet in the N terminal region of the CP or substitution of Thr by Ala at position 309 in the HC - pro of ZYMV.

Full length infectious clones of a severe strain of ZYMV were constructed and put under the control of a phage promoter, such as the T7 RNA polymerase promoter (Gal On A., Antignus Y., Rosner A., and Raccah B. (1991) *Journal of General Virology* 72:2639-2643), bacterial promoters or a promoter effective *in planta,* such as the cauliflower mosaic virus (CaMV) 35S promoter (Gal On A., Meiri E., Huet H., Hua W.J., Raccah B. and Gaba V. (1995) *Journal of General Virology* 76:3223-3227).

In the work presented here, the FRNK box is implicated, for the first time, as being of importance in symptom development, surprisingly without effecting the accumulation of the virus in the plant. Due to the highly conserved sequence of the FRNK box within the HC -Pro gene of the potyviruses, any substitution in the FRNK box of a potyvirus would have an effect on symptom development, not only the substitution of Arg in position 180 with Ile, in ZYMV, demonstrated in the work described here.

Based on the highly conserved genome, organization and gene function of the potyviruses, it may be concluded that the conserved FRNK box in the HC - pro gene has the same function in all potyviruses (perhaps as a receptor). Therefore, the substitution in the FRNK box in any of the potyviruses would have a similar effect on symptom development. Members of the potyviruses that are economically important are, for example, BCMV (Bean Common Mosaic Virus), BYMV (Bean Yellow Mosaic Virus), BtMV (Beet mosaic), MWMV (Moroccan watermelon mosaic), OYDV (Onion yellow dwarf), PRSV (Papaya ringspot), PStV (Peanut stripe), PepMoV (Pepper mottle), PVMV (pepper veinal mottle), CGVBV (Cowpea green vein banding), GEV (ground eyespot), ISMV (Iris severe mosaic), JGMV (Johnsongrass mosaic), LYSV (Leek yellow stripe), LMV (Lettuce mosaic), MDMV (Maize dwarf mosaic),

PPV (Plum box), PVA (Potato A), PVV (Potato V), PVY (Potato Y), SbMV (Soybean mosaic), SCMV (Sugarcane mosaic), SPFMV (Sweet potato feathery mottle), TEV (Tobacco etch), TVMV (Tobacco vein mottling), TBV (Tulip breaking), TuMV (Turnip mosaic), WMV-2 (Watermelon Mosaic Virus 2) , YMV (Yam mosaic), ZYFV (Zucchini yellow fleck).

The infectious clone may be an RNA transcript or a cDNA construct, though the use of infectious transcripts is the less efficient process i*n vitro*.

A method for providing protection against viral infection in plants, according to the present invention comprises inoculating plants with the recombinant potyvirus infectious construct, for example, by mechanical inoculation or by bombardment.

Compositions containing, as an active ingredient, the construct of the present invention may be used for superior plant cross protection, especially against infection by the severe strain of ZYMV and for transient expression of foreign nucleic acid in plants. The composition used for the introduction of the construct into plants, for infecting them by bombardment is an aqueous composition comprising, in aproximately equal volumes, the construct, a salt, such as calcium nitrate and particles such as tungsten, gold. The composition used for the introduction of the construct into plants by mechanical inoculation comprises infected plant tissue.

The construct of the present invention may be further used as a vehicle for the transient expression of foreign nucleic acid, namely genes, in a plant. The construct according to the present invention is highly infective, does not induce symptoms in the infected plants and is not transmitted by aphids.

Use of compositions, containing as an active ingredient, this clone provides an efficient, safe and environment friendly method for transient expression of foreign nucleic acid into the infected plants. Further applications of this construct may, therefore, be the expression of foreign sequences or genes within a defective RNA molecule of potyviruses. Defective RNAs are viral

RNA genomes which are missing some of the viral genes but which, together with a complete helper virus (the full length parental virus), can facilitate the expression of the sequences they contain. Defective RNAs are derived from the helper virus genome, but still require the presence of a complete helper virus for replication in the plant cell. The construct of the present invention may have viral genes removed from the full length clone and may then serve to support the expression of foreign genes via potyviruses defective RNA by co-infection of a plant with a full length clone, from which any viral genes are removed, together with a full length clone or virus harboring a full length clone.

A method for introducing foreign nucleic acid into plants according to the present invention comprises infecting a plant with a full length clone into which any sequence of DNA or RNA is inserted or co-infecting a plant with a full length clone, from which any viral genes are removed, together with a full length clone or virus harboring a full length clone.

A method for the production of a mild strain of potyvirus, according to the present invention comprises inoculating plants with the recombinant potyvirus infectious construct and collecting the resulting progeny.

The said invention will be further described and illustrated by the following experiments and figure. These experiments and figure do not intend to limit the scope of the invention but to demonstrate and clarify it only.

Brief Description of the Figure

Figure 1 is a schematic drawing of four constructs of ZYMV infectious cDNA (a-d).

Detailed Description of the Figure

Figure 1 is a schematic drawing of four constructs of ZYMV infectious cDNA (a-d). The open and striped bars indicate the ZYMV-NAT and ZYMV-WK sequences within the FLC respectively. The relevant restriction enzymes and

the amino acid changes are present. On the right side the severity of the symptoms in squash is indicated, from very severe (+++++) to mild (+). The sequence of the primer used for the mutagenesis is indicated.

Example 1 - full length clone (FLC) of ZYMV

Construction of the mutants in the full length clone (FLC) of ZYMV

The constructs which represent the HC - Pro sequences (Huet H., Gal On A., Meiri E., Lecoq H. and Raccah B.(1994) *Journal of General Virology* 75:1407-1414) of the ZYMV - WK strain were placed under the T7 RNA promoter in the infectious FLC. In order to get higher rate of infection with those constructs the fragment BstXI/AgeI from the FLC of 35SZYMVNOS cDNA (Gal On A., Antignus Y., Rosner A., and Raccah B. (1991) *Journal of General Virology* 72:2639-2643 and Gal On A., Meiri E., Huet H., Hua W.J., Raccah B. and Gaba V. (1995) *Journal of General Virology* 76:3223-3227), was replaced by the appropriate fragment from pZYHC (-) clone (Huet H., Gal On A., Meiri E., Lecoq H. and Raccah B.(1994) *Journal of General Virology* 75:1407-1414). Site directed mutagenesis was introduced on ssDNA template of the subclone pksM16B (Gal On A., Antignus Y., Rosner A., and Raccah B. (1991) *Journal of General Virology* 72:2639-2643), using the primer 5' ATGTTCATAAATAAGCGCTCTAG3' (amino acid Ile is underlined and the unique restriction site of Eco47III is in bold). The clone pksM16B carrying the mutations was double digested by BamHI/BstEII and the obtained fragment (1.4kb) was introduced to the same sites in the 35SZYMVNOS cDNA (Gal On A., Meiri E., Huet H., Hua W.J., Raccah B. and Gaba V. (1995) *Journal of General Virology* 76:3223-3227).

Plants, mechanical or bombardment inoculation and symptom appearance of the ZYMV AG1

Greenhouse - grown zucchini squash (*Curcurbita pepo*. L. cv Ma'ayan), cucumber (*Cucumis sativus* L. cv. Bet Alpha; Shimshon; Delila), melon (*Cucumis melo* L. cv. Arava) and watermelon (*Citrullus lanatus* Schad cv. Malali) plants were used at the cotyledon stage. The inoculated plants were maintained in a growth chamber under continuous light at about 25^{0}C. The plants were examined daily for visual symtom development.

Bombardment inoculation were as previously described by Gal - On et al. (1995). Mechanical inoculation of plants infected by the recombinant virus were performed by sap inoculation (100mg/ml), applied to a cotyledon previously dusted with carborundum.

Cross protection experiments

Cross protection by the ZYMV-AG1 strain was tested as described by Lecoq et al. (1991) Squash seedlings at the fully expanded cotyledon stage were bombarded with the 35S-AG1 at 0.1 g/ l. A week later they were infected in the greenhouse by 5 - 7 aphids (*Myzus persicae*) per plant according to Antignus Y., Raccah B., Gal - On A. and Cohen S. (1989) *Phytoparasitica* 17:287-289).

Determination of the mutation in the progeny virions

To ascertain the presence of the mutations in the viral RNA total mRNA from infected leaf tissue was extracted. The synthesis of the RT-PCR was performed as described by Huet et al. (1994).

ELISA assay for evaluation of ZYMV titer

Leaf discs of squash and cucumber ZYMV-infected plants were taken 7 - 10 d.p.i. and the homogenized tissue were subjected to ELISA as described by Antignus et al (1989).

Previously, sequence comparison has shown four amino acid changes in the 455 amino acid sequence of the HC - pro gene between the severe field strain (ZYMV - JV) and the mild field strain ZYMK - WK. The replacement of a fragment of the HC - Pro of ZYMV - WK containing two substitutions Aspartate (Asp) 148 and Arg 180 (BstXI/BstEII fragment), reduced symptom expression of the virus in squash plants without effecting virus accumulation. To distinguish which of the two substitutions, Asp 148 or Arg 180, effect symptom development, Arg 180 was replaced by Ile within the FRNK box (figure 1, clone d) by site directed mutagensis.

The engineered virus containing the Arg 180 replacement by Ile, was designated ZYMV-AG1. This new strain did not cause the development of symptoms in cucumber (three different varieties), melon and watermelon. The virus did accumulate to levels as high as that of the wild type ZYMV-JV. It was assumed, therefore, that the second amino acid difference (Asp at position 148) is dispensable for altering the symptoms from mild to severe.

In order to verify the presence of the amino acid changes within the mild virus ZYMV - AG1, and to prevent aphid transmission, a new restriction site of Eco47III was introduced at position 550 nt (from the 5' of the HC- Pro gene) and the DAG motif in the CP was replaced by DTG respectively (figure 1).

The new engineered virus (AG1) and a wild type severe strain (JV) accumulated to a similar level in systemically infected leaves of different curcurbit species (Table 1). Therefore, it may be concluded, that a point mutation changing amino acid Arg 180 to Ile, dramatically effects the severity of symtom development without effecting the movement and the replication of the ZYMV virus in the plant. The dramatic results confered by a point mutation in the potyvirus FRNK box, demonstrated in this work for the first time, could not have been inferred from the mere known sequence comparison which showed amino acid changes between the severe field strain and the mild field strain.

The stability of the amino acid substitution Arg 180 to Ile within ZYMV-AG1 was tested by infecting hundreds of squash plants and dozens of cucumber plants (Table 2). The presence of the Ile 180 mutation in the HC - Pro was confirmed by sequencing (data not shown). Curcurbit plants inoculated with ZYMV-AG1 mechanically or by particle bombardment with the ZYMV-AG1 strain did show the mild symptom appearance even throughout the growing period of the plant (Table 2). The presence of the Ile 180 mutation within the virion genome was confirmed by sequencing or indirectly by digestion of the RT-PCR amplified fragment with the restriction enzyme Eco47III (figure 1).

Replication and movement of the engineered ZYMV-AG1 strain remained high (as the wild type ZYMV), as seen from the accumulated level of the virus. These results suggest that no selective pressure is exerted to cause a reversion in the virus mutated genome.

The ability of the newly produced mild strain (ZYMV-AG1) to protect against a challenge inoculation of the severe strain of ZYMV (JV), was studied in cross protection experiments. Most of the protected plants did show mild symptoms after a challenge with the severe strain (96% protection). Two plants out of 47 that were infected with the ZYMV-AG1 strain and challenged a week later with the JV strain exhibited severe symptoms about one month post inoculation (Table 3).

The protection was studied in a small field experiment in which protected plants were exposed to field inoculation. Approximately 40% of the control non-protected plants became infected, while none of the protected plants showed severe symptoms. Therefore, no fruit damage was observed in the protected plants (Table 3). Previous studies showed that in a typical cross protection phenomenon, both the protective and the challenge virus strains are very closely related (Perring T.M., Farrar C. A., Blua M. J., Wang H.L. and Gonsalves D. (1995) *Crop Protection* 14 no. 7, 601 - 606). This is the first report where cross protection takes place between strains that have an identical

sequence, including the coat protein sequence, that differ only in a single amino acid in a non structural protein (the HC - Pro).

2) Cross protection in melons

Melon (*Cucumis melo* L. cv. Ofir) seedlings were planted and were infected with ZYMV-WK and the recombinant virus ZYMV-AG1. The viruses were sprayed onto the melon seedlings prior to planting. The seedlings were then planted together with untreated (control) seedlings.

Half of the plants at three weeks were challenged mechanically with the wild type virus (ZYMV-JV) and half were unchallenged for testing natural infection.

30 days after the begining of the experiment parameters such as the plant size and the extent of infection with the wild type virus, were studied. Plants infected with ZYMV-JV that were not treated by the weakened virus (WK) were small and showed clear infection symptoms. Plants treated with the recombinant virus (ZYMV - AG1) showed no symptoms of infection.

3) Expression of foreign genes through the ZYMV-AG1 clone in plants

For the expression of a foriegn gene in an infected plant, a Pst I site was inserted into the ZYMV-AG1 between the NIb and CP genes. The GFP (green flurocent protein) reporter gene and the Bar gene, which confers resistance to the non selective herbicide bialaphos (commercially named BASTA), were amplified by PCR, using primers containing the Pst I restriction site, and were inserted in the PstI site.

Plants were inoculated by bombardment with the ZYMV - AG1 containing the GFP reporter gene or Bar gene.

Biochemical analysis showed the GFP and Bar gene to be highly and stably expressed. Even after several passages, no revertants of the recombinant mild virus were found and the reporter gene and Bar expression remained high and stable. Plants expressing the GFP were luminecent and plants expressing the Bar gene were found resistant to the herbicide bialaphos.

Table 1. Comparison of virus accumulation between ZYMV-JV and ZYMV-AG1 strains in cucurbits.

experiment no.	no of tested plants: JV, AGI, WK	ZYMV-JV# severe ELISA OD(405)	ZYMV-AG1^ mild	ZYMV-WK~ mild
1s÷	11, 6, 6	0.9* (0.41)**	0.5 (0.19)	0.7 (0.18)
2s	2, 9, 8	1 (0.4)	0.7 (0.48)	-
3s	3, 10, 4	0.3 (0.08)	0.9 (0.27)	1.33 (0.13)
4s	9, 9, 9	0.51 (0.4)	0.46 (0.21)	0.59 (0.3)
5s	9, 9, -	0.56 (0.07)	0.7 (0.09)	-
6s	9, 8, -	0.82 (0.09)	0.95 (0.09)	-
7c	6, 7, -	0.7 (0.07)	0.81 (0.2)	-

Severe strain of ZYMV which found in Israel in the Jordan Valley (JV).

^ The engineered virus of ZYMV.

~ ZYMV weak strain described by Lecoq et al. (1991).

* Average of O.D (405) detected by ELISA from 11 plants.

** Standard deviation (in brackets).

÷ s and c are squash and cucumber test plants, respectively.

Table 2. The stability of the ZYMV-AGI virus in the plants

plant species	number of tested plants: bombardment with 35SAG1	* visual symptoms: mild	* visual symptoms: severe	# molecular analysis of Ilu-180 mutation
squash	402	398	0	10
cucumber	105	103	0	5
melon	30	30	0	3
Total	537^	531+	0	18

*Visual symptoms were observed and detected by ELISA about one and half month post inoculation.

The presence of the Ilu Mutation was confirmed by digestion of the RT-PCR by Eco47III restriction enzyme.

^ Total of bombarded plants.

+ Total of infected plants

Table 3. Cross protection in squash with the mild strain ZYMV-AG1 (induction) against the severe strain ZYMV-JV (challenge) in the greenhouse experiments.

	Number of tested plants				
experiment number*	induction ZYMV-AG1	#challenge ZYMV-JV	symptoms mild	severe	~fruit damage
a)	47	47	45	2	1
a)	14	-	15		0
a)	-	5		5	5
b)	15	15	14	0	0
b)	5	-	5		0
b)	-	5		5	5
c)	43	field inocul.	43		0
c)	-	6		6	6
c)18 healthy	-	field inocul.		7	7

* a, b and c are three separate experiments. a and b were in the greenhouse and c was done in a small plot in the field. c is a sum of two experiments where the protected plants (AGI) were exposed to field inoculation.

~ No. of plants showed fruit damage.

Inoculation by aphids.

CLAIMS

1) A recombinant potyvirus infectious nucleic acid construct useful for plant cross protection, comprising a full length clone characterized only in that its HC- Pro gene conserved FRNK box sequence contains a substitution.

2) A recombinant construct according to claim 1 wherein the nucleic acid is cDNA or an RNA transcript.

3) A recombinant construct according to claim 1 wherein the substitution in the conserved FRNK box is a substitution of Arg.

4) A recombinant construct according to claim 3 wherein Arg is substituted with an amino acid of the hydrophobic group or having a bulky side chain.

5) A recombinant construct according to claim 4 wherein Arg is substituted with Ile.

6) A recombinant potyvirus infectious nucleic acid construct according to claim 1-5 wherein the potyvirus is ZYMV.

7) A recombinant construct according to claim 6 wherein the construct is ZYMV-AG1.

8) A recombinant construct according to claim 1-7 further containing a substitution which effectively abolishes aphid transmissibility.

9) A recombinant construct according to claim 8 wherein the substitution which effectively abolishes aphid transmissibility is a substitution of the Ala

residue at position 10 in the conserved DAG triplet in the N terminal region of the CP.

10) A recombinant construct according to claim 7,8 and 9 useful for plant cross protection wherein the cross protection is against severe strains of ZYMV.

11) A recombinant potyvirus infectious nucleic acid construct according to claims 1-6 wherein the potyvirus is selected from BCMV, BYMV, BtMV, MWMV, OYDV, PRSV, PStV, PepMoV, PVMV, CGVBV, GEV, ISMV, JGMV, LYSV, LMV, MDMV, PPV, PVA, PVV , PVY, SCMV, SPFMV, TEV, TVMV, TBV, TuMV, WMV-2 , YMV and ZYFV.

12) A recombinant construct according to claims 1-11 further useful for the transient expression of foreign nucleic acid in plants wherein the full length clone has, in any position, a sequence of DNA or RNA inserted into the full length clone.

13) A method for providing protection against viral infection in plants comprising inoculating plants with the recombinant potyvirus infectious construct as defined in any of the preceding claims.

14) A method according to claim 13 wherein inoculating plants is done by mechanical inoculation or by bombardment.

15) A method for introducing foreign nucleic acid into plants comprising infecting a plant with a full length clone as defined in claim 11.

16) A method for the production of a mild strain of potyvirus comprising inoculating plants with the recombinant potyvirus infectious construct as defined in any of the preceding claims and collecting the resulting progeny.

17) A virus containing the recombinant construct as defined by claim 1 and as produced according to claim 16.

18) Produce inoculated with the recombinant construct as defined in the preceding claims and in the method according to claim 13.

19) Produce according to claim 18 wherein the produce are curcurbits.

20) Compositions for plant inoculation or for transient expression of foreign nucleic acid in plants containing, as an active ingredient, the recombinant construct according to claim 1-12 or a virus containing the recombinant construct according to claim 17.

ABSTRACT

The present invention relates to a recombinant potyvirus infectious nucleic acid, preferably cDNA or RNA, construct useful for plant cross protection, comprising a full length clone characterized in that its HC- Pro gene conserved FRNK box sequence contains a substitution, preferably a substitution of Arg. Preferably, the construct further contains a substitution which effectively abolishes aphid transmissibility. The present invention further relates to a method for the production of a mild strain of potyvirus utilizing the above mentioned construct and to a method for protecting plants from viral infection and for transient expression of foreign nucleic acid (genes) in plants, using said construct. Preferably, the present invention relates to a method for cross protection of curcurbits against ZYMV infection. The present invention also relates to a method for providing protection against viral infection in plants comprising inoculating plants, by mechanical inoculation or by bombardment, with the recombinant potyvirus infectious construct.

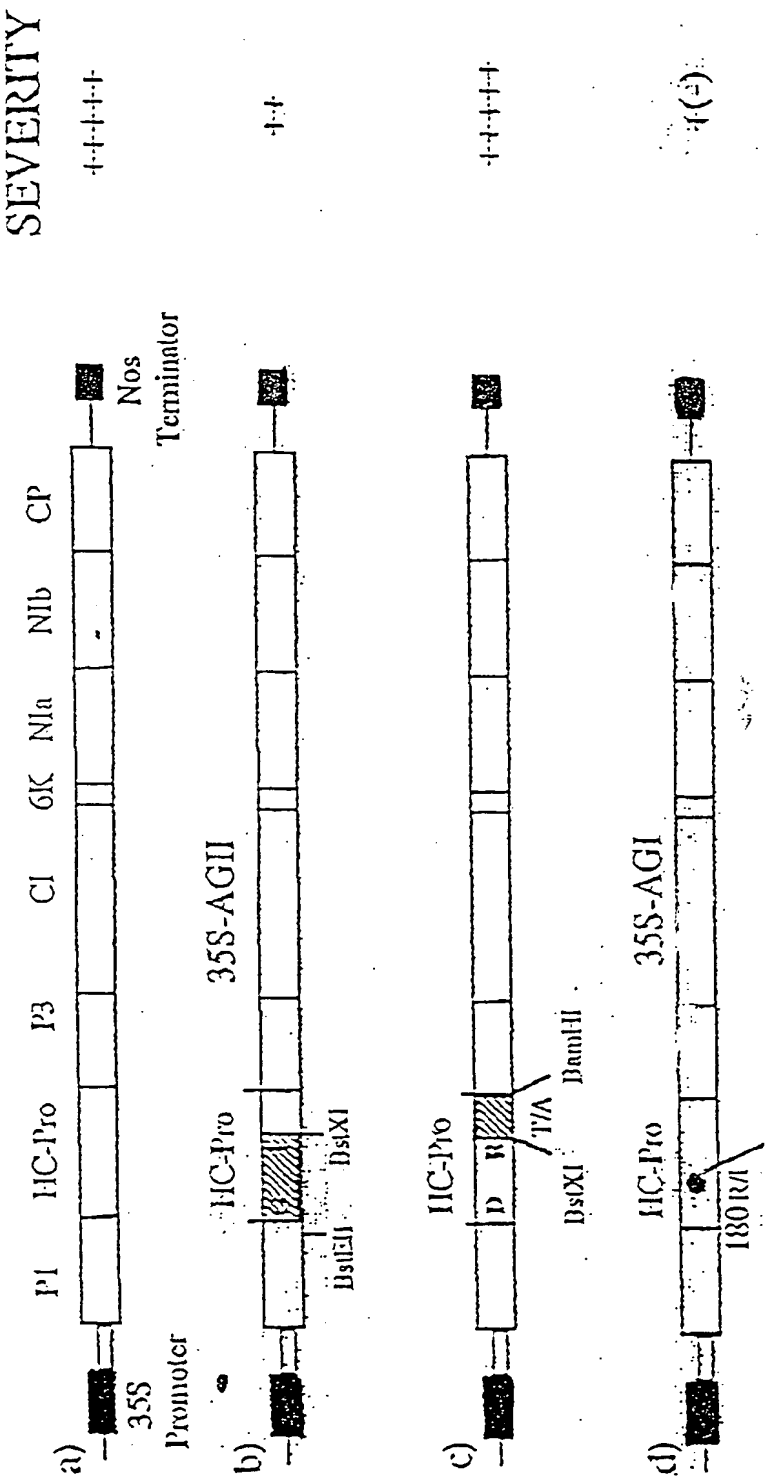
SEVERITY
a)
35S
Promoter
P1
HC-Pro
P3
CI
6K
NIa
NIb
CP
Nos
Terminator
b)
HC-Pro
35S-AGII
BstEII
BstXI
c)
HC-Pro
D
B
T/A
BstXI
BamHI
d)
HC-Pro
35S-AGI
180 bp
Eco47III
primer - 5'ATGTTCATAAATAAGCGCTCTAG-3'
IIa
Eco47III

FIGURE 1